HYPHAL TIP GROWTH: MOLECULAR COMPOSITION OF ELONGATING AND NON-ELONGATING REGIONS OF ACHLYA CELL WALL

By

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Although apical growth is a widespread process in the biological world and has been known for over a hundred years, the mechanisms that underlie this process are not yet understood. Knowledge of these mechanisms would allow the development of techniques for inhibiting or stimulating growth of medically or economically important species. I approached the problem of hyphal tip growth by comparing the cell wall composition of elongating and non-elongating regions of the oomycete *Achlya bisexualis*. Light microscope observations were used to determine the growth rate and to distinguish elongating and non-elongating hyphae for further EM studies, because non-elongating hyphae often are found among growing mycelia. I found that hyphal growth is a

discontinuous irregular process with periods of elongation and no elongation. The elongation rate is not steady, but instead fluctuates with periods of fast and slow elongation. Both transmission and scanning electron microscopes were used with a variety of cytochemical labels, and several fixation techniques. Cellulose, the microfibrillar component of the *Achlya* wall, was identified with cellulase enzyme-gold affinity labeling. Elongating hyphae have cellulose in mature and subapical regions, but not at the apex. In non-elongating hyphae, cellulose was found in all the regions including the apex. These results suggest that the apices of elongating hyphae lack cellulose. This contradicts the long-standing hypothesis that the microfibrilar component is present in the elongating hyphal apex. The 1,3-ß-glucans, the major matrix wall components, were immuno-localized in all regions of elongating and non-elongating hyphae. A number of cytochemical, biochemical and physiological controls were performed to assure the reliability of these findings. I suggest that in elongating regions, the matrix is synthesized first and synthesis of microfibrilar component follows. Another explanation for these results is that localized apical cellulose hydrolysis by endoglucanase creates plastic wall regions consisting mainly of 1,3-ß-glucans, which expand under turgor and/or cytoskeleton pressure. Cellulose deposition quickly follows to prevent "blowing out" of the hypha.

CHAPTER 1
INTRODUCTION

Apical Growth

Hyphal tip growth is a hallmark of the fungi, even though it also occurs in specialized plant cells (i.e., growth of pollen tube, root hair, moss protonema). Diverse animal cells share this capacity to protrude their cytoplasm and then move in that direction, a process termed ameboid movement. The essential feature of tip growth is that the tip of the hypha is protruded into the enviroment from the subapical region. This protrusion involves the synthesis and extension of the cell wall and cytoplasm (with its contained organelles). The organism is thus able to explore and exploit its environment. Although apical growth is a widespread process in the biological world and has been known for over a hundred years, the mechanisms underlying this process are not yet understood. Knowledge of these mechanisms would allow an understanding of other related characteristics of fungi, such as the influence of environmental factors on growth and morphogenesis and the interaction between fungi and other organisms. Ultimately, detailed knowledge of hyphal tip growth would allow the development of techniques for

inhibiting or stimulating growth of medically or economically important species.

Studying hyphal tip growth is a complex problem because the apex represents only a tiny part of a hypha. Most of the important growth events occur within 5 micrometers of the tip. On the other hand, the mature part of the hypha is not inactive. In growing hyphae, the wall synthesis per unit area is maximal at the tip. The total amount of wall material synthesized subapically at the same time is appreciable (Sietsma et al 1985). This also contributes to the difficulty of studying tip growth. Finally, not all of the hyphae in an actively growing colony are growing (apically elongating) at a given moment in time. Therefore, conventional biochemical, autoradiographical and cytological techniques must be adapted to the specificity of the problem.

In this study I approached the problem of hyphal tip growth by comparing cell wall architecture in elongating versus non-elongating hyphal apices of an oomycete *Achlya bisexualis*. Electron microscopy, both transmission and scanning, was used with a variety of immunocytochemical labeling of hyphae. Several fixation techniques were used to ensure that the results were not only reproducible but also not artifacts of the fixation procedure. The results allowed me to propose a new hypothesis for the mechanism of hyphal tip growth.

The Organism

Members of the genus *Achlya* grow as branched coenocytic hyphae, which collectively are termed a mycelium. Septa are formed only to delimit reproductive structures, while vegetative growth occurs at the apex. *Achlya* has both asexual and sexual cycles of reproduction. Asexual reproduction occurs by fragmentation, differentiation of resistant gemmae, or by the differentiation of vegetative apices into sporangia (Sparrow 1960). *Achlya* differs from related genera by the fact that the primary zoospores immediately encyst in a loose cluster at the orifice of the sporangium after discharge (Johnson 1956).

Sexual reproduction occurs by gametangial contact. The male gametes produced in an antheridium are transported via fertilization tubes to female gametes produced in an oogonium (Mullins 1994). Sexual morphogenesis is initiated and sequentially controlled by a series of diffusible steroid hormones (Raper 1939). While most water molds are monoecious, bearing both male and female reproductive structures on a single diploid mycelium, some members of the genus *Achlya* are dioecious. True "male" and "female" strains of dioecious species of *Achlya* may exist, but the expression of mating type in a

strain depends on that of its mating partner (Raper 1939). The involvement of hormones in sexual reproduction in this genus is very noteworthy, as species of *Achlya* appear to be the most primitive eukaryotes known to produce and respond to steroids.

Achlya has been proposed as a eukaryotic model system for studying basic mechanisms of growth and development. Species of *Achlya* have been used to investigate the regulatory mechanisms of steroid-hormone-induced and regulated sexual differentiation (Thomas and Mullins 1967, Mullins and Ellis 1974, Horgen 1977, Riehl and Toft 1984, Mullins 1994). They also served in studies on: (i) the differentiation of vegetative hyphae into asexual sporangia (Griffin and Breuker 1969, Thomas et al. 1974, LeJohn et al. 1977, Kropf et al. 1983, Cottingham and Mullins 1985); (ii) the mechanism of nutrient transport in fungi (Cameron and LeJohn 1972, Manavathu and Thomas 1982, Kropf et al. 1984); (iii) the tropic responses to nutrients and other chemoattractants (Musgrave et al 1977, Manavathu and Thomas 1985); (iv) the role of turgor in hyphal tip growth (Money and Harold 1992, 1993); and (v) ionic and electrical currents (Harold 1994). In this study, I used *Achlya bisexualis* Coker and A. Couch (ATCC accession number 14524) to investigate the mechanisms of hyphal tip growth.

Class Oomycetes

The genus *Achlya* is classified in the family *Saprolegniaceae*, order *Saprolegniales*, class *Oomycetes*, subdivision *Mastigomycotina*, division *Eumycota* of the kingdom *Fungi* (Carlile and Watkinson 1994). The subdivision *Mastigomycotina* contains organisms that produce motile spores (zoospores). The subdivision is divided into three classes, based on the morphology of zoospores: *Chytridiomycetes, Oomycetes,* and *Hyphochytriomycetes*. The first class is similar to other *Eumycota*, while the latter two show similarities to some protists rather than to fungi. In fact, the morphological divergence of the *Oomycetes* has long been recognized based on their morphology (Gaumann and Dodge 1928). Their biochemical properties, such as L-lysine biosynthesis (Vogel 1964), cell wall chemistry (Bartnicki-Garcia 1968), and tryptophan-pathway enzyme organization (Hutter and DeMoss 1967) strongly support this view. More recent ultrastructural (Beakes 1987) and molecular studies (Lovett and Haselby 1971, Ohja et al. 1975, Kwok et al. 1986, Forster and Coffey 1990, Forster et al. 1990) also confirmed the divergence of the *Oomycetes*. According to Bartnicki-Garcia (1996), these biochemical and morphological differences indicate that the *Oomycetes* and the higher fungi probably arose from different ancestors. However, the same author disagrees with the idea of breaking up the kingdom *Fungi* based on these phylogenetic

considerations. In the past, the classes *Chitridiomycetes*, *Oomycetes*, and *Hyphochytriomycetes* often have been grouped with nonfungal organisms with which they have very little in common, either on a physiological, morphological, or ecological basis. For example, the *Oomycetes* were lumped with heterokont algae in the kingdom *Chromista* (Cavalier-Smith 1983, Moore-Landecker 1996), or were placed with all zoosporic fungi, protozoa and algae in kingdom *Protoctista* (Margulis et al. 1990). An admittedly polyphyletic kingdom *Fungi* is a more rational taxonomical solution than the ones listed above. This solution allows us to assemble and study the collection of organisms that share key morphological, physiological and ecological properties (Bartnicki-Garcia 1996).

Though my work does not concern systematics, an understanding of the phylogenetic position of *Achlya* is relevant to the problem of hyphal tip growth. Because the *Oomycetes* could have evolved independently, their mechanism of hyphal tip growth, despite its superficial similarity to one of true fungi, could prove to be different.

There are about 600 species of the *Oomycetes*. The sexual phase of the *Oomycetes* has a clear differentiation into large female and small male structures, termed oogonia and antheridia. These are the sites of meiosis and gametogenesis. Each oospore produced after

fertilization has a single diploid nucleus. When the oospore germinates, it gives rise to a diploid vegetative mycelium, in contrast to the haploid mycelium of most fungi. Other characters of the *Oomycetes* that distinguish them from the *Eumycota* are the biflagellate zoospore; mitochondria with tubular cristae; Golgi bodies consisting of multiple flattened cisternae; cellulose as a microfibrillar component of the cell wall; the presence of the amino acid hydroxyproline in cell wall glycoproteins; and various other biochemical and molecular characteristics (Carlile and Watkinson 1994). True fungi have mitochondria with platelike cristae and produce Golgi bodies that are very simple in structure, often consisting of only a single cisternal element. Cell walls of true fungi have chitin as the microfibrillar component and do not contain hydroxyproline (Alexopoulos et al. 1996).

The class *Oomycetes* consists of 5 orders: *Saprolegniales*, *Lagenidiales*, *Peronosporales*, *Rhipidiales* and *Leptomitales* (Alexopoulos et al. 1996). The order *Saprolegniales* contains a single family *Saprolegniaceae*. Usually these fungi occur in fresh water and in soil as saprotrophs and play an important role in decomposition and recycling of materials in aquatic ecosystems. Some, however, are obligate parasites of plants, animals, or other fungi. For example, some species of *Saprolegnia*, *Achlya*, and *Aphanomyces* attack fish and their eggs

(Alexopoulos et al. 1996). The members of *Saprolegniaceae* are often called water molds, are distributed universally, and are among the easiest fungi to isolate and cultivate in the laboratory.

CHAPTER 2
LITERATURE REVIEW

Mechanism of Apical Growth

The phenomenon of hyphal tip growth has been known for over a hundred years (Reinhardt 1892). Its mechanism, though, is not yet understood. Several theories of hyphal tip growth dominate the literature. They are (1) the delicate balance theory (Park and Robinson 1966, Bartnicki-Garcia 1973); (2) the steady-state theory (Wessels 1990); and more recently a combination of the first two, (3) the hybrid theory (Johnson 1996). All three imply that the wall of the apex is plastic, while that of the subapical nongrowing area is rigid. They also assume that the driving force for cell elongation is turgor pressure and/or cytoskeleton.

The delicate balance theory assumes that the plasticity of the hyphal apex is achieved by a constant delicate balance between biosynthesis and hydrolysis of wall components.

The steady-state theory suggests that the plastic region at the tip contains a mixture of nonlinked wall polymers that are being constantly synthesized, and the rigid condition of the wall is established by chemical crosslinking that is initiated at or near the tip and

continues progressively further back in the hyphal wall. Presoftening of the apical wall is catalyzed by endolytic enzymes that briefly initiate growth but do not sustain it.

The hybrid model retains from the steady-state model the constant exocytosis of a plastic mixture of wall polymers at the tip and its rigidification via crosslinking. Among the concepts retained from the delicate balance model is continuous endoglycanolytic activity expressed in proportion to the rate of tip extension (Johnson 1996).

Thus these models suggest different mechanisms to explain the events of wall growth, while agreeing on other aspects such as the role of turgor and the cytoskeleton.

Structure of the Hyphal Wall

Fungal cell walls have essential roles in the life of the fungal cell, i.e., maintenance of cell shape, plasticity, protection against unfavorable environmental conditions, cellular recognition, immune response, and host-parasite interaction (Rosenberger 1976, Wessels and Sietsma 1979). The general organization of hyphal cell walls comprises an inner layer of microfibrillar polysaccharides overlaid by an outer layer of amorphous polysaccharides (Burnett 1979).

In *Oomycetes*, these polysaccharides are, respectively, cellulose and 1,3-ß-glucans containing some

1,6-ß branches. Cellulose usually represents about 20% (w/w), 1,3-ß-glucans about 80% (w/w) of the total wall carbohydrates (Sietsma 1969, Burnett 1979).

In the hyphal wall of *A. ambisexualis* Raper (Reiskind and Mullins 1981a), acid-soluble 1,3-ß-glucans with single 1,6-ß-linked residues as branches represents 40% (w/w) of the dry wall. An alkali-soluble glucan, a polymer of 1,3-ß and 1,4-ß linkages with occasional 1,6-ß glucosyl residues as side chains, represents 7% (w/w) of the wall. Cellulose represents 21% (w/w) of the wall. An insoluble residuum with a linkage pattern similar to the alkali-soluble fraction is present at 6% (w/w). An insoluble component consisting of glucosamine represents 3% (w/w) of the wall. This insoluble fraction probably represents chitin (Mullins et al 1984). Protein containing hydroxyproline residue comprises 10% (w/w). There is also a small amount of phosphorus.

In the study on the ultrastructural organization of the hyphal wall of *A. ambisexualis* (Reiskind and Mullins 1981b) a model of the various layers in the wall was proposed. The method used in this study of the hyphal wall consisted of the sequential chemical or enzymatic removal of the various fractions, followed by analysis (with electron microscopy) of carbon-platinum replicas. The model shows (a) a surface layer of amorphous 1,3-ß-glucan hydrolyzed by acid or the enzyme laminarinase; (b) another 1,3-ß-glucan layer containing

some 1,4-ß and 1,6-ß linkages hydrolyzed by alkali or laminarinase; (c) microfibrillar cellulose, removed by cadoxen or the enzymes cellulase plus protease; and (d) an innermost layer of insoluble residuum, faintly microfibrillar.

The most abundant and most thoroughly studied glucans from the fungal cell walls are ß-glucans. These 1,3-ß-glucans are variable in degree of 1,6-ß branching and in the length of the branches.

Cellulose is a linear polysaccharide made of glucosyl moieties joined through 1,4-ß linkages. The glucan chains in this polysaccharide associate through hydrogen bonding to form microfibrils. According to chain orientation, different crystalline structures exist. The most prevalent form is Cellulose I, where glucose chains are arranged in parallel fashion (the free reducing groups are in the same end of the microfibrils, and the nonreducing ends are in the opposite one). In this sense, as demonstrated by X-ray diffraction analysis (Reiskind and Mullins 1981a), and also apparently in size, fungal cellulose is similar to the polysaccharide found in plants (Ruiz-Herrera 1991).

Chitin is an unbranched polysaccharide containing exclusively N-acetylglucosamine residues linked 1,4-ß. Three crystalline isoforms of the polysaccharide exist in nature, according to the arrangement of the chains. These forms can be recognized by X-ray diffraction. In fungi,

only alpha-chitin, characterized by the antiparallel arrangement of the chains, has been detected (Sentandreu et al. 1994).

Structural proteins present in the cell wall of fungi are glycoproteins. They display a basic common structure, consisting of protein with covalently bound carbohydrate chains. In fungi, they are usually called mannoproteins because the carbohydrate moiety mainly consists of mannose units, although small amounts of other sugars and phosphodiester groups are found (Peberdy 1990, Ruiz-Herera 1991). Hydroxyproline is reported as a constituent of cell wall proteins in the *Oomycetes* (Webster 1980, Reiskind and Mullins 1981a, Ruiz-Herrera 1991). The carbohydrate moieties are attached to the protein through two types of linkages. One type is *O*-glycosidic linkage between mannose or small oligosaccharide chains and the hydroxy-amino-acids (Nakajima and Ballou 1974, Sentadreu and Northcote 1969, Tanner and Lehle 1987). The second type of linkage (*N*-glycosidic) connects high molecular weight, highly branched, mannan tufts to asparagine residues of the protein, through diacetylchitobiose (Byrd et al. 1982, Cohen and Ballou 1981, Tanner and Lehle 1987).

Studies of the structure of fungal cell walls by cast-shadowing or replica techniques have demonstrated that their outer and inner surfaces appear different. The outer surface is usually amorphous or finely granular,

whereas the inner face shows intertwining microfibrils of different size, width and orientation. However, there is evidence that some components such as microfibrils may escape observation because they are masked by the presence of the large amounts of matrix compounds. From a structural point of view, the fungal cell wall has been compared to such manmade composites as reinforced concrete or fiber-reinforced plastics which are formed by two distinct elements: an elastic one, which in the cell wall would be microfibrils of the structural polysaccharides, and a plastic one, which would correspond to the rest of the wall components, generally referred as amorphous or cementing (Ruiz-Herrera 1991).

Electron Microscopic Studies of Fungal Cell Walls

In thin sections, fixed and stained by the usual standard method including glutaraldehyde and osmium tetroxide, fungal cell walls appear multilayered. At least two layers are observed in most walls: an outer one which is electron dense; and an inner layer, thicker and electron transparent. However, appearance of the cell wall in sections may depend on the technique used for fixation (Ruiz-Herrera 1991).

Variability in composition of the cell wall of fungi does not allow the proposal of a single model of the wall structure. In general, evidence suggests that fibrillar polysaccharides are accumulated mostly in the inner layers of the cell walls, while amorphous components are

more abundant in the external layers. The description of wall structure observed in different genera of fungi analyzed by various techniques may be more useful in providing a general overview of fungal wall architecture (Ruiz-Herrera 1991).

Early studies on the chemical characterization of fungal cell wall layers were conducted by Hunsley and Burnett (1970). They studied the wall structure of *Neurospora crassa, Schizophyllum commune* and *Phytophtora parasitica* after treatment with several hydrolytic enzymes. The outer surface of *N. crassa* in shadow-cast samples appeared amorphous. Laminarinase treatment removed the amorphous coat revealing a layer of coarse strands whose interstices were filled with amorphous material, whereas treatment with both laminarinase and pronase enhanced the reticular appearance. The microfibrils were sensitive to chitinase. The authors concluded that the external coat was made of amorphous beta-glucans placed over a reticulum of glycoproteins. More internally, it was suggested, a protein layer followed in which chitin microfibrils were embedded. In contrast to *Neurospora,* the cell wall of *S. commune* was resistant to laminarinase, pronase and chitinase, apparently due to the presence of superficially located 1,3-alpha-glucan which prevented the access of the lytic enzymes. After removal of this glucan layer by KOH, laminarinase and pronase treatment gave rise to the

appearance of a fibrillar structure sensitive to chitinase, suggesting that inner wall layers had a chemical composition and organization similar to *N. crassa*. Appearance of the wall from *P. parasitica* was not affected by pronase, but laminarinase unmasked a fibrillar layer sensitive to cellulase treatment. These results were interpreted as suggesting the presence of two layers rich in amorphous beta-glucans and cellulose, respectively, in the wall of this fungus.

The cell of yeast and mycelial cells of *Candida albicans* reveals four wall layers when treated by a standard gluteraldehyde-osmium technique (Yamaguchi 1974). When stained by Thiery's technique, eight different layers can be observed, depending on the intensity of staining and their electron density. The four outermost layers are PATAg positive, whereas layers 5 and 7 appear electron transparent and PATAg negative (Poulain et al. 1978). The authors concluded that the inner layers must be rich in chitin and 1,3-ß-glucan, which are both electron transparent and PATAg negative. Other outer layers must be rich in glucans and mannans. The existence of mannans on the surface of the cell was confirmed by Horisberger et al. (1975) who observed binding of colloidal gold-tagged concanavalin A (ConA-Au) by intact cells of the fungus. The presence of mannan in two continuous layers at the periphery of blastospores was demonstrated by staining ultrathin sections with

Concanavalin A-horseradish peroxidase-3,3'diamino benzidine and H0 (Tronchin et al. 1979). In this technique, the lectin binds to the mannose residues of the glycoprotein and it is recognized by peroxidase. The peroxidase forms a dark product by the catalytic decomposition of HO in the presence of an oxygen acceptor. A similar method, which included treatment with wheat germ lectin followed by chitibiosyl-horse radish peroxidase or chitobiosyl-ferritin, was used to conclude that chitin was located mostly in the inner layers of the wall of *C. albicans*.

In related species *Candida utilis*, sections were stained with ConA-Au and gold-labeled antimannan antibodies. These techniques demonstrated that mannoproteins were denser in the cell periphery although labeling also was observed close to the plasmalemma (Horisberger and Vonlanthen 1977). Similar results were obtained with *Saccharomyces cerevisiae* by the same authors (Horisberger and Vonlanthen 1977).

Lectins bound to fluorescein isothiocyanate (FITC) were used to detect superficial polysaccharides in the different yeasts by Barkai-Golan and Sharon (1978). The authors observed that *S. cerevisiae, S. bayanus* and *Candida mycodema* bound ConA only, suggesting the presence of mannoproteins on the surface of the cells. On the other hand, *Schizosaccharomyces pombe* did not bind ConA; but it bound peanut lectin, which recognizes D-galactose,

indicating that the cell surface of fission yeast is covered by a galactomannan, not by mannoproteins. *Candida rugosa* and *Sporobolomyces roseus* bound both lectins. This result may indicate the presence of both galactomannan and mannoproteins on the surface of these cells. Treatment of the cells with KOH resulted in a strong reaction with wheat germ lectin which recognizes GlcNAc, suggesting that chitin is located internally and is covered by alkali-soluble mannoproteins. The presence of galactomannan on the surface of *S. pombe* was confirmed by use of the lectin from *Bandeiraea simplicifolia* bound to colloidal gold (Horisberger and Rosset 1977). This lectin, which recognized alpha-galactopyranosyl residues bound to the outer layer of the wall, and in minor amounts was distributed evenly over the whole thickness of the cell, including the fission scars. In a further report, these authors demonstrated differential distribution of galactomannan depending on the growth stage of the cells (Horisberger et al. 1978). Galactomannan appeared in the form of two layers of the wall: one close to the plasmalemma, and another on the surface of the cell. Labeling by the lectin occurred at the cell periphery and at the growing end, but not on the wall, formed after cell division. These results were interpreted as meaning that the polysaccharide was synthesized during cell extension, but not during septum formation.

Four layers in the cell wall of *Dictyostelium discoideum* spores were observed by freeze-etching and replica (Hemmes et al. 1972). The innermost layer, which appeared amorphous or slightly fibrillar, could be eliminated by successive treatment with cellulase and pronase, suggesting that it was constituted by a mixture of cellulose and proteins. The middle layers (both fibrillar) were removed by cellulase treatment alone, indicating the cellulosic nature of the microfibrils. The most superficial layer was resistant to both pronase and cellulase treatment. Hydrolysis resulted in release of galactose, suggesting that this is a major component of the acidic polysaccharide present in the walls.

In sections of *Agaricus bisporus* spores treated with the standard method, three layers could be recognized. The authors concluded that the middle layer contained protein because treatment with pronase increased the fibrillar appearance of this layer. These fibrils corresponded to 1,3-ß-glucans and chitin, as they were removed by ß-glucanase and chitinase treatment. The outer layer was composed of melanins and 1,3-alpha-glucans, which was deduced by chemical analyses and electron microscopic observations. The thin inner layer was poorly characterized, but the authors suggested it was of mucilagenous nature (Rast and Hollenstain 1977).

The structure of the mycelial wall of the same fungus was different (Michalenko et al. 1976). The outer

layer, which appeared amorphous in replicas, was made of mucilage. The thin middle layer was made of amorphous glucans. The innermost layer, which in replicas appeared fibrillar, is probably made of a mixture of ß-glucans covering fibrillar chitin, since chitinase by itself could not remove it, whereas the combined action of ß-glucanase and chitinase solubilized the layer. Staining with silver hexamine suggested that proteins were present in all layers of the cell wall of the fungus.

A similar approach was followed in the characterization of the architecture of the wall from microconidia of *Trichophyton mentagrophytes*. Three layers were recognized in sections. The outer layer appeared electron dense, and the innermost one appeared electron transparent. The material extracted from the outer layer contained a single glycoprotein. The median layer was made of proteinaceous rodlets. The inner layer apparently was composed of amorphous glucans and microfibrillar chitin (Wu-Yuan and Hashimoto 1977).

Structure of the cell wall from *Trichoderma pseudokoningii* was studied by treatment of intact cells with different lytic enzymes (Jeenah et al. 1982). Accordingly, the authors concluded that the outer layer contained ß-glucans, whereas the internal layer was composed of chitin embedded in a protein matrix.

Biogenesis of the Fungal Cell Wall

Cell wall biosynthesis takes place in three sites: cytoplasm, plasma membrane and the wall itself. Structural polymers such as chitin and 1,3-ß- and 1,4-ß-linked glucans are synthesized vectorially at the plasma membrane, by transmembrane synthases accepting nucleotide sugar precursors from the cytosol and extruding the polymerized chain into the wall (Cabib et al. 1983, Shematek et al. 1980, Girard and Fevre 1984, Jabri et al. 1991, Cabib et al. 1991, Hromova et al. 1989). Matrix polymers such as glycoproteins are synthesized in the cytoplasmic secretory pathway of endoplasmic reticulum through Golgi vesicles to secretory vesicles. Wall assembly, involving activities such as covalent crosslinking of polymers and modifications such as deacetylation of chitin, takes place in the wall itself (Gooday 1995).

Fungal wall 1,3-ß-glucans are biosynthesized via the nucleotide sugar, UDP-glucose. The glucan synthases are intrisic proteins of the plasma membrane. Preparations of membranes from *Saprolegnia monoica*, when provided with UDP-glucose, produce polymers containing varying amounts of 1,4-ß and 1,3-ß links (Girard and Fevre 1984). The vectorial synthesis of 1,3-ß-glucan chains allows only linear molecules to be made and thus any 1,6-ß branches must be added in the wall (Gooday 1995). These

1,3-ß-glucan synthases are stimulated by the presence of trypsin but inhibited by other proteases. Stimulation occurs from the beginning of incubation in the presence of the protease but prolonged action of trypsin leads to inactivation of the glycosyl transferases. The 1,3-ß-glucan synthases, therefore, must exist in an inactive state that can be activated by moderate proteolysis. Such regulation, which also appears to modulate plant glycosyl transferases (Girard and Maclachlan 1987), characterizes the chitin synthase system of various fungi (Cabib 1981).

The 1,4-ß-glucan synthases, like 1,3-ß-glucan synthases, have a transmembrane orientation in the plasmalemma, leading to a vectorial synthesis of cellulose from UDP-glucose. These enzymes may have a common structure or organization, as revealed by preliminary immunological studies, but they are different systems and can be separated by glycerol-gradient centrifugation (Fevre et al 1990). Cellulose synthases from *Saprolegnia* are inactivated by trypsin, but stimulated in the presence of certain nucleotides. Fungal cellulose synthase enzymatic complex may resemble the plant plasma membrane rosettes involved in cellulose synthesis (Mullins and Ellis 1974, Muller and Brown 1980, Montezinos 1982). Some proteins, sensitive to proteases or capable of reacting with nucleotides, would be involved in the regulatory processes. Other proteins

would be involved in UDP-glucose binding. Such an enzyme seems to exist in plants (Delmer 1999). Cellulose synthases may have a more complicated organization than 1,3-ß-glucan synthases. Cellulose synthase activities of cell free extracts are always much lower than 1,3-ß-glucan synthase activities. It is possible that cellulose synthases require a specific factor that is lost in the course of isolation. The opposite behavior of the synthases towards protease and nucleotides, and the presence of a membrane bound activator of 1,3-ß-glucan synthase, may indicate a difference in the regulation of their activities. This would have implications in the cell wall assembly where the deposition of the different polysaccharides during apical growth is coordinated in time and space (Fevre et al 1990).

Chitin synthases, like glucan synthases, are intristic proteins of plasma membrane. These enzymes catalyze glycosidic bond formation from the nucleotide sugar substrate, UDP-N-acetylglucosamine. Most chitin synthase preparations are zymogenic, i.e., produced as proenzymes requiring activation by specific proteases. This proteolytic activation presumably plays a role in the temporal and spatial regulation of the enzyme, by locally activating it in the membrane when and where its activity is required (Gooday 1995). As well as being in zymogenic and active forms in the plasma membrane, zymogenic chitin synthase also occurs in fungal cells as

chitosomes, which are membrane-bound microvesicles about 70 nm in diameter (Bartnicki-Garcia et al 1979, Kamada et al 1991). After purification by differential centrifugation, chitosomes can be activated by treatment with proteolytic enzymes, and then produce chitin microfibrils if incubated with UDP-GlcNAc (Gooday 1995).

Wall glycoproteins are biosynthesized in the secretory pathway: endoplasmic reticulum > Golgi bodies > secretory vesicles > release at the plasma membrane. Carbohydrate material detected in apical vesicles could be the carbohydrate portion of glycoproteins. The transmembrane stages in glycoproteins biosynthesis involve sugar precursors linked to polyprenol dolichol, the "lipid intermediates" (Lehle 1981, Cabib et al. 1988). In the O-linked chains, the first mannose unit is linked to the protein via the precursor dolichol-phosphomannose, in the endoplasmic reticulum. The other mannose units are added via the nucleotide sugar guanosine diphosphomannose, GDP-Man, in the Golgi bodies. The N-linked chains are assembled by a more complex scheme, giving a lipid intermediate dolichol-diphospho-(GlnNAc)2-Man9-Glc3 which is N-linked to asparagine in the protein in the endoplasmic reticulum, with the release of the terminal four sugar units, Man-Glc3. The outer chain of many mannose units is added by several linkage-specific mannosyl transferases, with Glc-Man as substrate, in the Golgi bodies (Gooday

1995). Some secreted enzymes, notably invertase, acid phophatase and chitinase, are also mannoproteins, synthesized and secreted in a similar fashion (Kuranda and Robbins 1991).

Once the cell wall components are synthesized and secreted, they must be converted into an integrated structure. This process includes covalent crosslinking, hydrogen bonding, hydrophobic and electrostatic interactions between different macromolecules (Ruiz-Herrere 1991).

Role of Turgor in Wall Expansion

The difference in hydrostatic pressure between a cell and its surroundings is called turgor pressure. This actual pressure is thought to provide the driving force for hyphal extension. Several observations and measurements suggest that it is necessary for the apical growth process (Robertson 1958, Park and Robinson 1966, Robertson and Rizvi 1968). Osmometry has been used to demonstrate a correlation between hyphal extension rates and turgor pressure in many fungal species (Eamus and Jennings 1986, Luard and Griffin 1981, Woods and Duniway 1986). Experiments have shown that filamentous fungi respond to increases in external osmotic pressure by accumulating compatible solutes, including potassium ions, glycerol, mannitol, erythritol and arabitol (Luard 1982a,b,c; Pfyffer and Rast 1988, 1989).

The most detailed analyses of the relationship between hyphal extension and turgor pressure have been carried out on hyphae of *Achlya bisexualis* and *Saprolegnia ferax* and these studies suggest that growth can occur without significant turgor (Money and Harold 1992, Kaminskyj et al. 1992). The rate of growth under these conditions is about half of the maximum rate. *Achlya* continues to grow even after the turgor is undetectable; however, its morphology is radically altered. On solid medium it shows plasmodial-like growth. In liquid medium of the same composition, it exhibited a yeast-like morphology. *Saprolegnia* has a different response to the absence of turgor, since it continues to grow in the hyphal form. Both *Achlya* and *Saprolegnia* appear not to respond to changes in external osmotic pressure by controlling the concentration of internal compatible solutes (regulation of turgor); instead, the plasticity of the wall is modulated to balance the force applied against it.

Role of Cytoskeleton in Hyphal Growth

It seems very unlikely that the thin wall covering the apices of extending cells has sufficient mechanical strength to contain the turgor pressure of the cytoplasm. It was suggested that other cellular components may play a role in the regulation of tip expansion. The possible existence of other factors is suggested by: (1) the ability of mutants with abnormal cell wall composition to

generate relatively normal hyphae (Katz and Rosenberger 1970); (2) the poor correlation between growth rates and turgor pressure (Kaminskyj et al. 1992); and (3) the ability of some species to produce hyphae in the absence of measurable turgor pressure (Money and Harold 1993).

The apex might be stabilized by a fibrillar cytoplasmic network similar to that found in other cellular systems (amoebae, slime molds). The main structural components of such a cytoskeleton are actin filaments (F-actin), microtubules, and intermediate filaments. Each of these are elongated polymers composed primarily of globular proteins known as actins, tubulins, and other unrelated units respectively (Heath 1994). The presence of an array of F-actin was shown in growing tips of *Saprolegnia* (Heath 1987). It was always present in growing tips, but absent in nongrowing tips. None of these observations proves a morphogenic role for F-actin, because it is not possible to differentiate between direct and indirect effects in the complex system of hyphal tips, but they do suggest that F-actin has some role (Heath 1994).

Another explanation of the presence of actin plaques at the growing apices is vesicle and organelle traffic control. There is evidence for the involvement of both microtubules and F-actin in wall vesicle transport (Heath 1994).

Cytology of Growing Hyphal Apices

A growing hypha consists of an apical region where the extension takes place, a nonelongating subapical, and mature regions, which were the sites of earlier growth. This is reflected both in the structure of the wall and the cytoplasm.

Older regions of the hyphal wall are rigid and thick. The cytoplasm in these regions is restricted to a thin layer between a large tonoplast and the plasma membrane. This cytoplasm contains the usual variety of eukaryotic organelles. The tonoplast in mature regions is represented by a central vacuole whereas younger regions contain many vacuoles of smaller size. The subapical region has a thinner wall. The cytoplasm is nonvacuolated and is particularly rich in organelles. At the very apex, the hyphal wall is thin and the associated cytoplasm lacks the usual organelles, containing only small cytoplasmic vesicles of differing size (Shapiro 1995). Based on the organelle distribution, three cytoplasmic zones or regions are recognized: (1) an older, highly vacuolated region; (2) a subapical organelle-rich region; and (3) a terminal vesiculate region (Grove et al 1970).

Since one of the differences between the growing tip region and the basal parts of the hypha is the abundance of cytoplasmic vesicles, they are usually assumed to be involved in the synthesis of the new wall. One possibility would be that they carry wall polymers ready

for insertion in the growing wall. Intracellular synthesis of wall polymers and their delivery to the wall by vesicles occurs with pectin, hemicellulose, and hydroxyproline-rich glycoproteins in plants (Northcote 1984), and to wall mannoproteins in yeast (Zlotnik et al. 1984). For filamentous fungi, there is no convincing evidence for a similar process. Cytochemical staining does detect polysaccharide material in some apical vesicles (Grove 1978, Hill and Mullins 1980), but this material may represent glycoproteins destined for secretion. More likely, these vesicles contain precursors of the cell wall, their membrane probably contributes to the extending plasmalemma, and they may contain wall synthase enzymes for insertion into the plasma membrane (Heath 1994).

Growing and nongrowing hyphae differ in the type of wall material that covers their apices (Wessels 1986). The absence of alkali-insoluble beta-glucans at the very apex of growing hyphae in *Schizophyllum commune* has been demonstrated by light microscopic autoradiography (Wessels et al. 1983). A subsequent study using electron microscopic autoradiography on shadowed preparations revealed that chitin in growing apices, though alkali insoluble, is in a conformation state quite different from that in nongrowing apices and subapical parts. In contrast to the chitin in these older parts, the newly synthesized chitin at apices appeared nonfibrillar, very

susceptible to chitinase degradation and partly soluble in hot dilute mineral acid. Earlier observations had indicated discontinuities in the presence of microfibrils at hyphal apices (Strunk 1968). These have been contradicted by other workers who showed a continuous network of chitin microfibrils over the apex after chemical treatments which removed a "matrix substance" (Aronson and Preston 1960, Hunsley and Burnett 1970, Bartnicki-Garcia 1973, Schneider and Wardrop 1979, Burnett 1979, Aronson 1981). Wessels (1990), however, suggested that these images showing apical microfibrils probably represent nongrowing apices, which are known to occur abundantly among growing hyphae.

There is a number of light microscopic studies using fluorescently labeled probes which also suggest that the wall covering the growing apex is different from that covering a nongrowing apex or that of subapical regions. In these studies fluorescently labeled antibodies (Fultz and Sussman 1966, Marchant and Smith 1968, Hunsley and Kay 1976), fluorescent brighteners such as calcofluor (Gull and Trinci 1974), and fluorescently labeled wheat germ agglutinin were used. This differential staining at growing tips could result from the absence of outer wall materials, or to a difference in the conformation of the polymers that bind these probes.

Calcium Gradient

There is a tip-high calcium gradient in apically growing cells. Free cytoplasmic calcium in the oomycete *Saprolegnia ferax* is highest at the tip as demonstrated using fluorescent dyes such as Indo-1 or Fluo-3 (Yuan and Heath 1991, Jackson and Heath 1993, Garrill et al. 1993). Studies using patch-clamp techniques suggest that the tip-high gradient reflects a spatial organization of calcium channels in the cell membrane. Using patch-clamp electrophysiology, two types of channels were identified in *Saprolegnia ferax*: (a) calcium-activated potassium channels that were thought to be involved in turgor regulation, but were not obligatory for growth; and (b) stretch-activated calcium channels that were activated by potassium ions and which may be essential for apical extension (Garrill et al. 1992, 1993). The stretch-activated channels were concentrated at the hyphal apex and were blocked by Gd3 which also inhibited hyphal extension and dissipated the tip-high calcium gradient revealed by Indo-1 (Garrill et al. 1993). In contrast to the stretch-activated channels, the calcium-activated potassium channels were uniformly distributed along the hyphal cell membrane. These could be inhibited by tetraethylammonium, which only caused a transient effect on growth. Stretch-activated calcium channels have also been identified in the germ tubes apices of the plant pathogen *Uromyces appendiculatus*

(Hoch et al. 1987, Zhou et al. 1991). These data suggest that the tip-high calcium gradient is important for polarized hyphal extension and is generated by a locally high concentration of stretch-activated calcium channels in the hyphal apex. It is presumed that the channels are delivered to the surface in microvesicles. They may be maintained there by anchoring them to the cytoskeleton or by membrane recycling (Gow 1995).

Ion Currents

The net flow of electrical current carried by the circulating ions can be detected with an ultrasensitive voltmeter called the vibrating microelectrode (Jaffe and Nuccitelli 1974). In *Achlya bisexualis* and filamentous fungi in general, a positive proton-carried current normally enters the growing apex. Inward current was shown to be due to amino acid-proton co-transport (symport) localized at the tip and the outward current was due to electrogenic proton efflux via a plasma membrane ATPase (Kropf et al. 1984, Gow et al. 1984, Gow 1984, Schreurs and Harold 1988). The proton current also established an extracellular pH gradient around the hypha, with the medium adjacent to the tip relatively alkaline (Gow 1984). On the other hand, there are many examples where there is no correlation between the direction or magnitude of the current and the process of tip growth (Gow 1995).

CHAPTER 3
HYPHAL GROWTH

Introduction

Until recently, hyphae were assumed to extend at a constant linear rate when environmental factors are favorable and stable, and nutrients are ample. Detailed analysis of hyphal growth, however, reveals oscillating elongation rates (Lopez-Franco et al. 1994).

Materials and Methods

An isolate of *Achlya bisexualis* Coker & A. Couch (ATCC 14524) was used in this study. Stock cultures were maintained on corn meal agar. Mycelia were grown on corn meal agar (CMA), prepared from 17 g of Difco corn meal agar, 10 g of purified grade agar (Fisher Scientific) and 1 L of distilled water. Small plugs (approximately 1 millimeter) were removed from the edge of a 24-hour-old CMA colony. The plugs were then placed in a 250 mL flask containing 100 mL of liquid peptone-yeast extract-glucose (PYG) medium, pH 6.8 (Cantino and Horenstein 1953). The PYG medium was prepared by combining 1.25 g of bacto-peptone, 1.25 g of yeast extract (Sigma) and 3 g of D-glucose with 1 L of distilled water. The culture was incubated for 10 to 12 h until the hyphae had grown out

from the agar plugs to a distance of 2 to 5 mm and then studied.

To obtain actively growing colonies, the 10 to 20 h old cultures incubated in PYG were used. Based on dry weight accumulation, colony size increase, glucose incorporation, and cellulase secretion, these colonies are in the midexponential stage of growth (Hill and Mullins 1979).

Non-growing conditions were obtained by transferring growing colonies from PYG to 0.2% glucose solution, by incubation in glucose for about 48 h to cease elongation. Procedure made it possible to find colonies with no elongating hyphae. Such colonies were fixed and used for studying non-elongating hyphae. Screening for no elongation is necessary, because there are hyphae in some colonies that are still elongating at a slow rate.

Hyphal elongation was monitored with an Olympus BH-2 light microscope. The colonies were kept on small depression slides with cover slips. Digital images of the hyphal tips were taken every 10 min with a Pixera 120C digital camera. The microscope light was turned off between the measurements to avoid heating. Average elongation rates were calculated using a stage micrometer. One hundred hyphae from different growing colonies and about 50 hyphae from non-growing colonies were studied.

Results

These light microscopic observations suggested that in colonies growing in PYG, the majority of the hyphae are elongating and a small number of the hyphae is not. When individual hyphae are monitored over a long period of time (5 to 6 h), they go through alternating periods of elongation and non-elongation. The rate of elongation is not steady, but fluctuates between periods of fast and slow rates. The average rate is 3.6 µm/min, but it fluctuates from 2 to 6 µm/min. The fastest rates are in the middle of an elongating cycle, with lower rates at the beginning and the end, resulting in a bell shaped curve (Fig. 1). Elongating hyphae have sharp apices (Fig. 2).

The majority of the hyphae in the colonies incubated in glucose-only medium are not elongating and a small portion of the hyphae (about 5%) is elongating with an average rate of 1 µm/min. In some colonies there are no elongating hyphae at all. All the hyphae have rounded apices (Fig. 3).

Discussion

Light microscopic observations suggest that hyphal growth is a discontinuous, irregular process with periods of elongation and no elongation (Fig. 1). The elongation rate is not constant, but instead fluctuates with periods of fast and slow elongation. During the elongation period the higher rates are in the middle and the rate changes

in a bell shaped curve mode. A similar irregular mode of hyphal tip growth was demonstrated by Lopez-Franco et al. (1994). Growing hyphal tips were recorded with video-enhanced phase-contrast microscopy at high magnification, and digital images were measured at very short time intervals (1 TO 5 s). The study was conducted using fungi from several major taxonomic groups (Oomycetes, *Pythium aphanidermatum* and *Saprolegnia ferax*; Zygomycetes, *Gilbeltella persicaria*; Deuteromycetes, *Trichoderma viride*; Ascomycetes, *Neurospora crassa* and *Fusarium culmorum*; Basidiomycetes, *Rhizoctonia solani*). In all fungi, apparent steady growth of hyphal tips revealed patterns of pulsed hyphal elongation. It was shown that the hyphae do not grow continuously with a steady rate but instead this rate fluctuates, with alternating periods of fast and slow elongation. This results in irregular pulses of growth. Pulsed growth was observed in fungi differing in cell diameter, overall growth rate, taxonomic position, and presence and pattern of Spitzenkorper organization, thus suggesting that it is a general phenomenon. The basis of these pulses was not determined, it was proposed that their origin could be in the pulsating mode of intracellular processes, especially the secretory vesicle delivery/ discharge system.

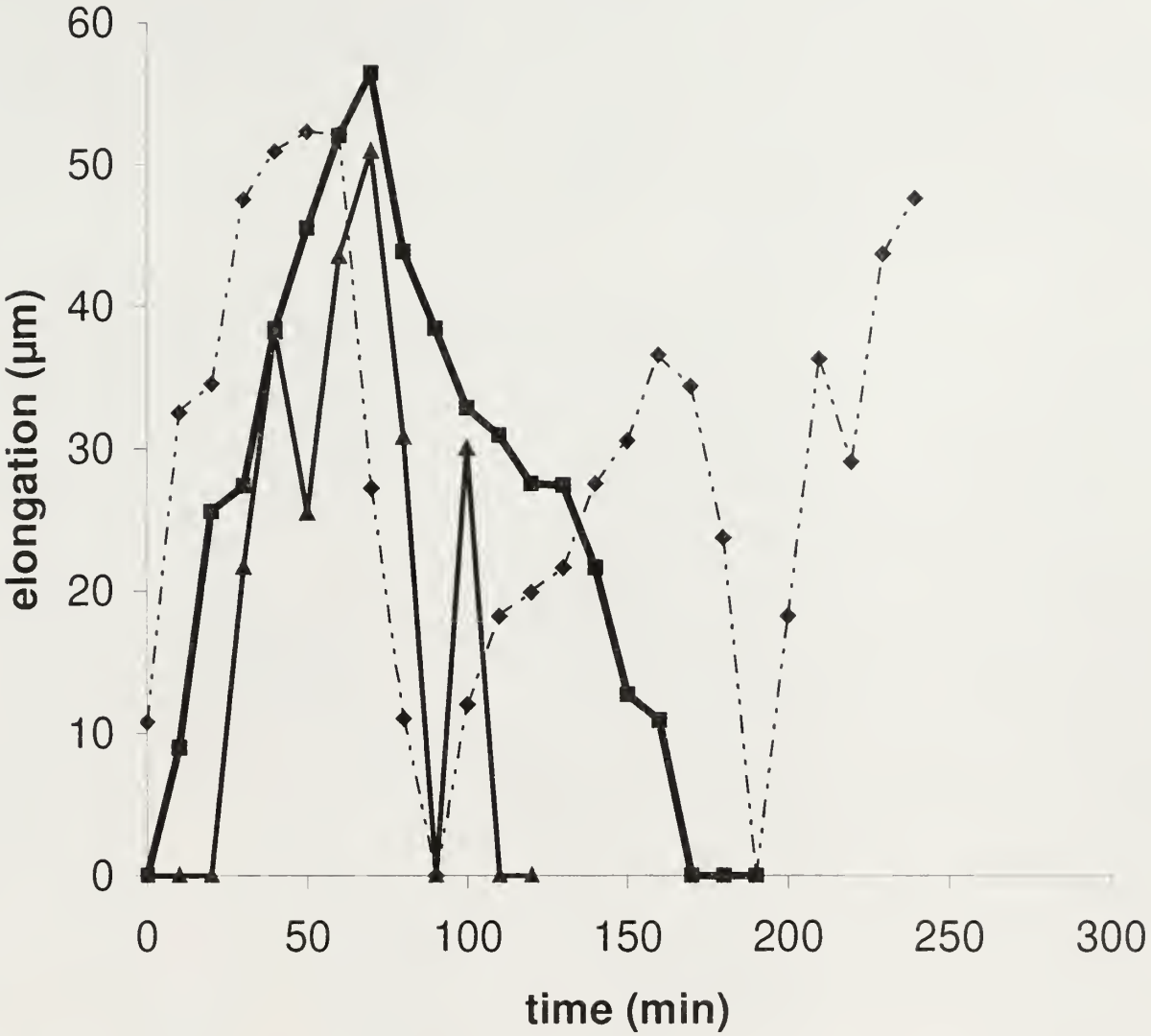
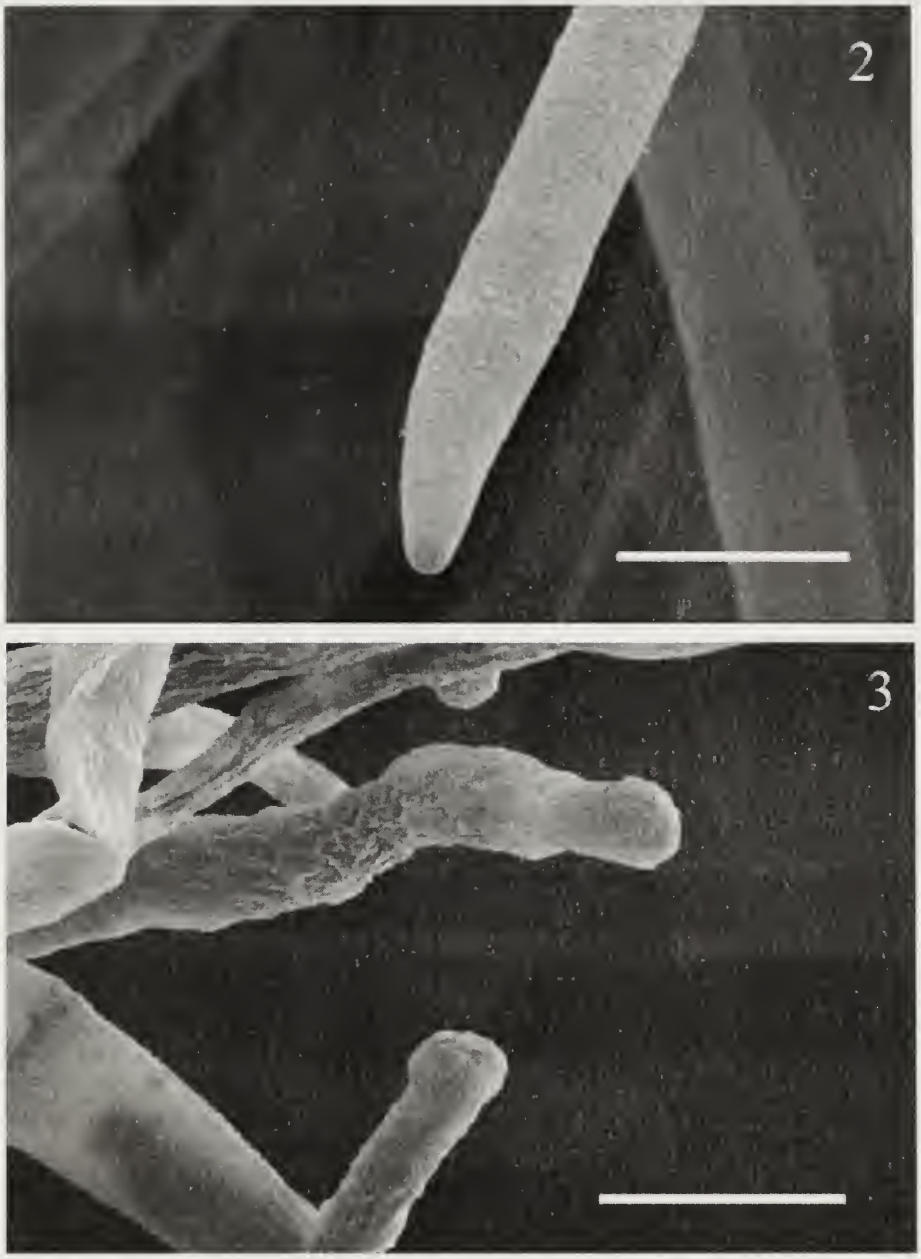


Fig. 1. Elongation measurements of three individual *Achlya bisexualis* hyphae growing in PYG medium.



Figs. 2-3. Scanning electron micrographs of hyphae of *Achlya bisexualis*. 2. Elongating hypha from a colony incubated in PYG. 3. Non-growing colony incubated in glucose-only medium showing the hyphae with rounded apices. Bars: 2 = 16.7µm; 3 = 27.3 µm.

CHAPTER 4
LOCALIZATION OF CELLULOSE IN THE CELL WALL AS REVEALED BY ELECTRON MICROSCOPY AND CYTOCHEMICAL TECHNIQUES

Introduction

Cellulose, a crystalline 1,4-ß-glucan, is the most abundant biopolymer in nature. Its biomass makes it a global carbon sink and renewable energy source, and its crystallinity provides mechanical properties to cellulose containing cell walls (Arioli et al. 1998). The understanding of cellulose properties and metabolism is important for understanding morphogenesis in plants and certain fungi.

Traditionally, cell wall components have been identified cytochemically by using indirect, extractive methods. This approach can lead to problems such as incomplete extraction and unseen effects of the extraction procedure on cell wall ultrastructure. Enzyme-linked colloidal gold labeling is a nondestructive, direct labeling technique and can be used to localize cellulose on thin sections (Berg et al. 1988). This labeling technique can be also used to localize cellulose on the surface of cells.

Previously cellulose was identified in *Achlya* cell walls with cellulase-gold affinity labeling. Thin

sections of growing hyphae revealed labeling for cellulose in mature and subapical regions, but not at the apex (Shapiro 1995).

The present study was done to observe the distribution of cellulose along the elongating and non-elonging hyphae, as part of an overall examination of hyphal tip growth.

Materials and Methods

Culture Methods and Microscopy Techniques

The general culture methods are described in Chapter 3. To induce sporulation, ten discs from the edge of a 48 h old colony growing on CMA plates were punched out with a small cork border. The discs were incubated at room temperature in 100 mL PYG liquid medium in a 250 mL flask for 15 h with shaking at 110 rpm. Then they were washed several times with 0.5 mM calcium chloride. At ths point, they were left in fresh calcium chloride for 6 to 7 h to induce sporulation.

Electron microscopy

For chemical fixation, the agar plugs bearing hyphae were fixed for 30 min at room temperature with 4% (v/v) glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2. After rinsing in 3 changes of buffer, the material was postfixed in 1 % (w/v) osmium tetroxide in the same buffer, for 30 min. Samples were again washed several times in buffer, followed by dehydration in an ethanol series, terminating in absolute acetone. For

freeze-substitution fixation colonies were frozen in acetone at -80 C, then substituted with methanol at -80 C for 72 h. The samples were warmed over 2 h at room temperature and transferred into absolute acetone for TEM or rehydrated in a methanol/water series to be labeled with cellulase-gold complex and processed for SEM (modified from Bourett et al. 1998). For methanol fixation colonies were frozen in methanol at -80 C, then warmed at room temperature over 2 h and transferred into absolute acetone for TEM or rehydrated in an methanol/water series. These samples were labeled with cellulase-gold complex and processed for SEM. Material from absolute acetone was infiltrated with an epoxy embedding medium and polymerized at 60 C for 48 h in a flat embedding mold. Epon 812 embedding medium was prepared by combining 55 g of Epon 812, 35 g of DDSA and 21 g of NMA. The accelarator DMP-30 (0.2 mL per 10 mL of the medium) was added right before embedding. Embedded samples were sectioned on a Reichert Ultracut R (Leica). Thin sections (75 to 80 nm) were collected on formvar coated nickel grids and labeled with the cellulose-gold complex.

For scanning electron microscopy the colonies were fixed with 4% glutaraldehyde in 0.05 M sodium cacodylate buffer and washed several times in buffer (osmium tetroxide fixation was omitted). Then the colonies were processed for cellulase-gold labeling, silver enhanced,

dehydrated in an ethanol series finally critically point dried. For silver enhancement the colonies were placed in a non-diluted mixture (1:1) of reagents from the Aurion Silver Enhancement Kit for 5 min and washed several times with water to stop the reaction (Scopsi et al. 1986).

Cellulose Localization Using Enzyme-Gold Affinity Labeling

Colloidal gold of approximately 15 nm diameter was made via reduction of chloroauric acid by sodium citrate as described by Frens (1973). The enzyme cellulase was purchased from Worthington Biochemical Corporation, catalogue No. LS02601. This is chromatographically "purified" cellulase isolated from cultures of a selected strain of *Tricoderma reesei*. A second enzyme was also used, endocellulase III (provided by Dr. Tim Fowler, Genencore International, Inc). The solutions used for conjugation with this enzyme were 5.5 rather than 4.5 for the commercial cellulase. To coat the gold with cellulase, the pH of 10 mL of 15 nm colloidal gold was adjusted to 4.5 and then 1 mg of cellulase dissolved in 0.1 mL distilled water was added with stirring. After 5 minutes the enzyme-gold complex was further stabilized by the addition of 0.5 mg/mL polyethylene glycol (molecular weight 20,000). Then the solution was poured into a centrifugation tube and 1.5 mLof 20% glycerol (in citrate buffer pH 4.5) was carefully placed on the bottom of the tube (glycerol was added for long-term storage at -80 C).

The enzyme-gold complex was pelleted at 12,100 rpm for 1 h. Successful coating was evident by a mobile pellet, which was resuspended in 0.75 mL of 20% glycerol.

Sections, on grids, were preabsorbed for 5 min by floating them face down on citrate buffer containing 0.5% gelatin as a blocking agent. The labeling solution was a 1:10 dilution of the enzyme-gold stock with citrate buffer. To label, grids with sections were floated on the labeling solution for 30 min. The grids were then floated on citrate buffer alone for 5 min and rinsed twice for 5 min in distilled water.

The colonies destined for SEM observations were treated with the same series of solutions, but were completely submerged, rather than floated.

Cytochemical controls

A number of cytochemical controls were performed to prove the specificity of the label. (1) Substrate competition: as a control to determine that the enzyme-gold probe was binding to cellulose the cellulase-gold complex was incubated with 1 mg/mL carboxy-methylcellulose (CMC) (sodium salt, medium viscosity, Hercules CMC 7MF) for 30 min before the labeling of sections or colonies. (2) Labeling with nonenzymatic protein: any nonspecific protein binding sites were determined by incubating the sections and colonies with 18 nm Colloidal Gold-AffiniPure Goat

Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.). (3) Substrate specificity check: two substates similar to cellulose and present in the cell wall of *Achlya* were tested to see if cellulase-gold bound nonspecifically to them: the cellulase-gold complex was incubated with 10 mg/mL laminarin (from *Laminaria digitata*, Sigma) and 11.8 mg/mL re-acetylated glycol chitosan (Sigma). (4) Cellulase pretreatment: to determine the effect of predigestion by free cellulase the sections and colonies were incubated with 1 mg/mL cellulase in incubation buffer for 30 minutes prior to labeling with cellulose-gold complex.

Cellulase Enzyme Activity during Labeling

To determine if the cellulase enzyme, when conjugated to gold used for labeling, retained enzyme activity the following experiment was done. The samples were combined with 1 mL of gold-cellulase complex (1:10 dilution) and incubated at room temperature. A control for each sample was prepared with substrate and 0.05 M sodium citrate buffer pH 4.5. The citrate buffer alone was used as a blank. After 30 min (the usual time of labeling) and 3 h, the enzyme activity was checked by Nelson-Somogyi method, using a standard curve obtained by plotting optical density (at 520 nm) measured on Du-64 Spectrophotometer (Beckman) against known concentrations of glucose. The samples included: 1 mg CMC, 1 mg of the whole *Achlya* wall, small colony and 1 mL of

cellulase-gold complex alone.

Detection of Gold Particles with a Backscatter Detector

To assure that the particles observed on the hyphal surfaces were actually the gold particles, the regular secondary images were compared with the backscattering images of the same regions. The samples were carbon coated, instead of the usual gold coating. Backscatter detector (GW Electronics, USA) was used to detect the backscattering signal.

Zymolyase Hydrolysis

Chemically fixed or live colonies were incubated at room temperature with 0.05 mg/mL zymolyase 100 T (*Arthrobacter luteus*) (Seikagaku Corporation) in 66 mM sodium phosphate buffer pH 7.5. The hydrolysis was monitored with light microscope. After 24 h the colonies were fixed, labeled with cellulase-gold complex and processed for SEM observations.

Treatment of Growing Colonies with Dichlorobenzonitrile

Small colonies were grown in 500 mL flasks containing 250 mL of PYG. Each flask contained 10 small colonies. After 12 h of incubation, dichlorobenzonitrile (DCB) was added to the flasks. DCB was previously dissolved in 100% DMSO. The concentrations of DCB were 10, 20, 30, 40, 50, 60 100, and 200 µM. The colonies were incubated in DCB-containing medium and their growth was monitored with light microscopy. After 36 h of incubation, the colonies were chemically fixed, labeled

with cellulase-gold complex and processed for SEM observations. The colonies grown in PYG only medium served as a control in this experiment. The ability of spores to germinate in the medium containing DCB was also checked. For this 5 mL of fresh spore suspension were added to PYG medium with and without DCB.

Results

Cellulose Localization

In growing colonies (incubation in PYG) cellulose is found on the surface of mature and subapical regions on the hyphae. In apical regions three patterns of labeling are found in a single colony. Some of the hyphae (approximately 5%) are not labeled at the apex and show a sharp border between labeled and non-labeled regions. The unlabeled area is approximately 2 to 4 μm in diameter (Figs. 4-11). In the majority of the hyphae, there is a gradual decrease of the labeling towards the apex (Figs. 12-19). Some of the hyphae (about 5%) are labeled at the apex as intensively as in the mature regions (Figs. 20-27). Fifty colonies from different batches were examined and all of them had this pattern and ratio of labeling.

In contrast, the labeling of hyphae from colonies incubated in glucose-only medium gave a different pattern of cellulose labeling. In these colonies, all the hyphae were labeled at the apices and the labeling was as intensive as in mature regions (Figs. 28-35). Ten

colonies from different batches were analyzed. Light microscopic analysis prior to fixation ensured that they did not contain elongating hyphae.

The surface labeling of hyphae in the freeze-substituted and methanol-fixed colonies has the same patterns and ratio as in chemically fixed ones (data not shown).

Cellulase-gold affinity labeling of cross sections localizes cellulose in the cell wall exclusively (Fig. 36). The distribution of the gold particles in the wall is even. The level of nonspecific labeling is very low. On the longitudinal sections of elongating hyphae the label is present in mature and subapical regions but is very low or absent in apical regions (Fig. 37).

There is no labeling on the cross sections (Fig. 38) or the hyphal surface (data not shown) when the sample of endocellulase III from Dr. Fowler was used. The conjugation was successful, based on the raspberry red color of the enzyme-gold complex and the presence of the mobile pellet. Negative staining of the enzyme-gold complex confirmed successful conjugation (Fig. 39).

Cytochemical controls

All the cytochemical controls support the view that the cellulase-gold complex is a specific label for cellulose in *Achlya*. The preabsorbtion of the labeling solution with CMC results in the absence of the labeling

on the hyphal surface as well as on the cross sections (Figs. 40, 41).

The incubation of the sections and the colonies with gold-coupled Goat Anti-Mouse IgG results in the absence of labeling as well (Figs. 42, 43).

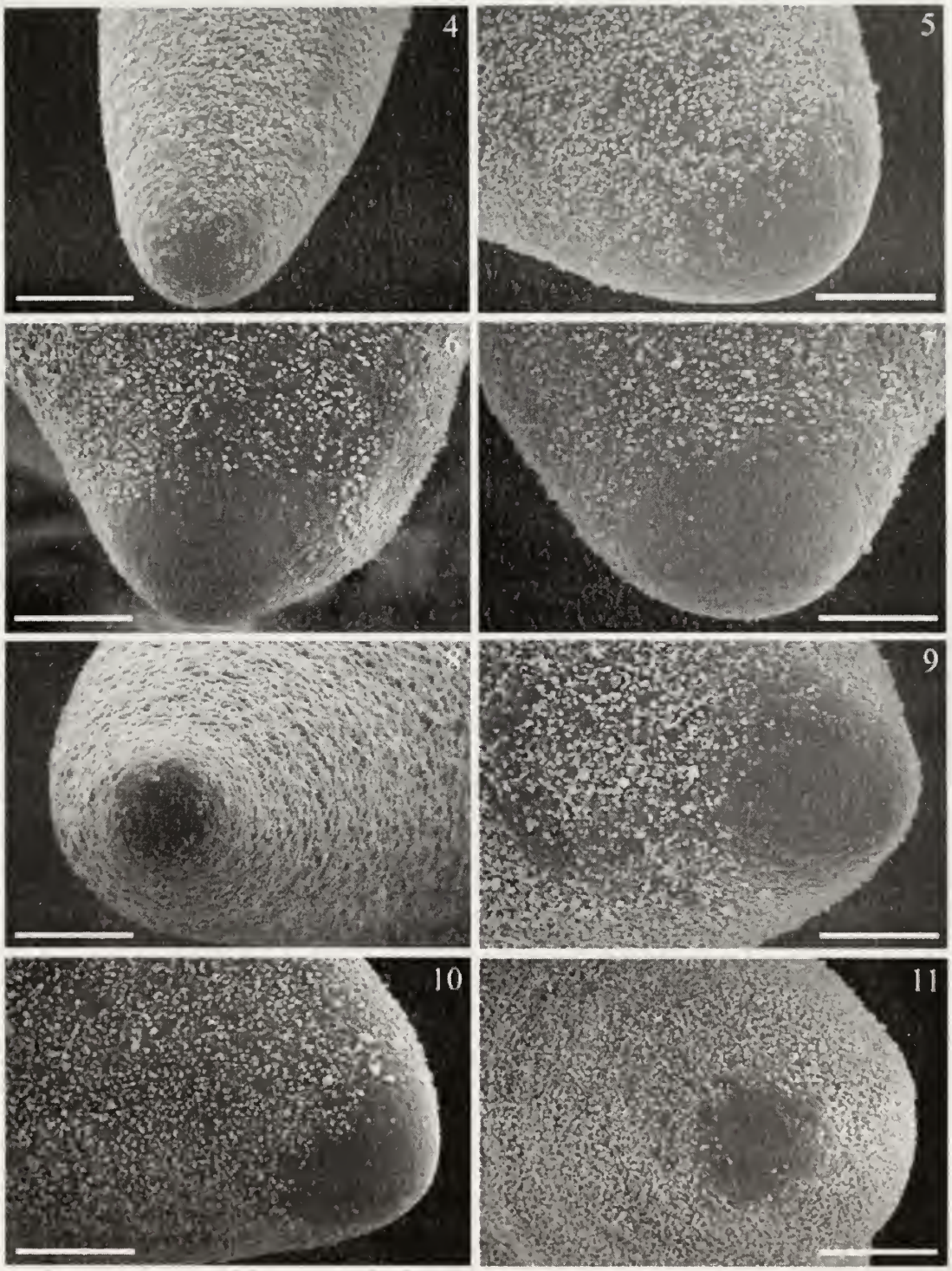
Cellulase pretreatment of the sections and colonies results in the absence of the labeling (Figs. 44, 45).

The labeling pattern is regular when the cellulase-gold complex is incubated prior to the labeling with laminarin or chitosan (Figs. 46-49).

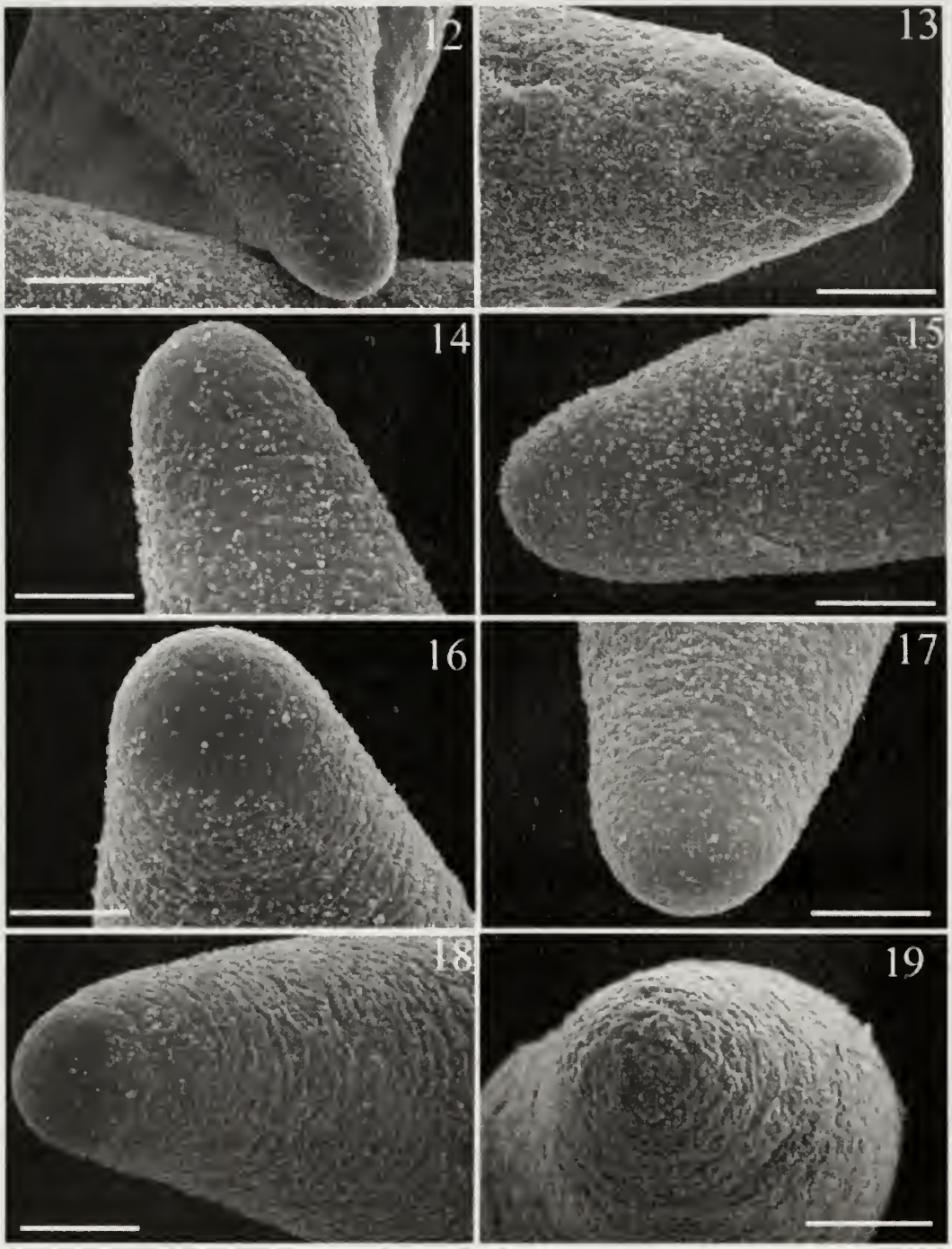
Detection of gold particles on the hyphal surface with a backscatter detector gave an identical particle distribution on secondary and backscatter images (Figs. 50-55).

Cellulase activity during labeling

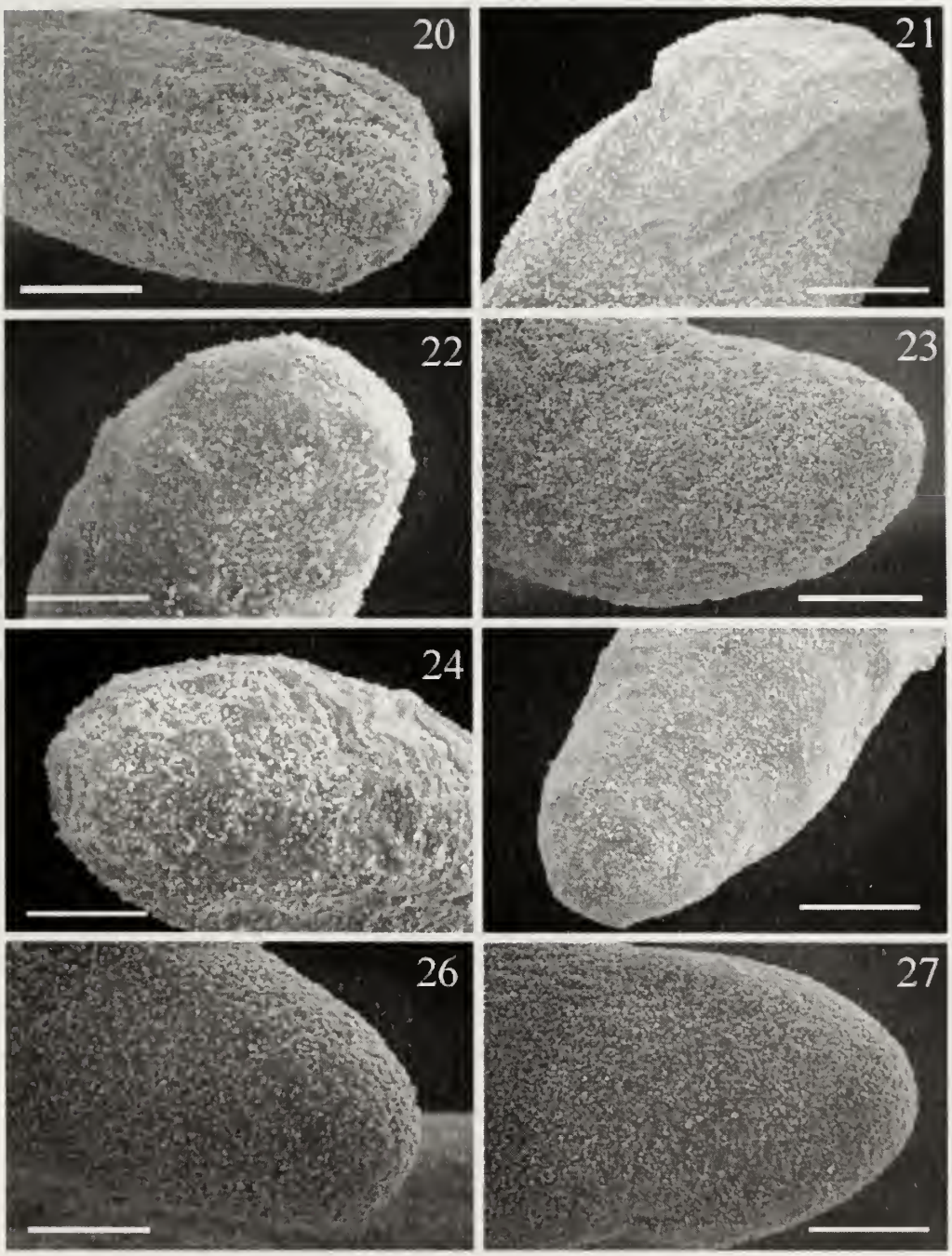
Cellulase-gold complex shows no enzyme activity during the labeling of the colonies or whole wall samples as measured by the production of reducing sugar (Table I). Absorbance of glucose is measured after 30 min (the usual time of labeling with cellulase-gold complex) and 3 h. Cellulase-gold is diluted 1:10 with the buffer. Based on glucose equivalents from a standard curve, cellulase activity is very low in reactions with a whole wall preparation or a colony. Thus, there are no additional primers produced and they do not alter the results of labeling. Enzyme activity of cellulase-gold complex against cellulase itself is also low. Cellulase activity



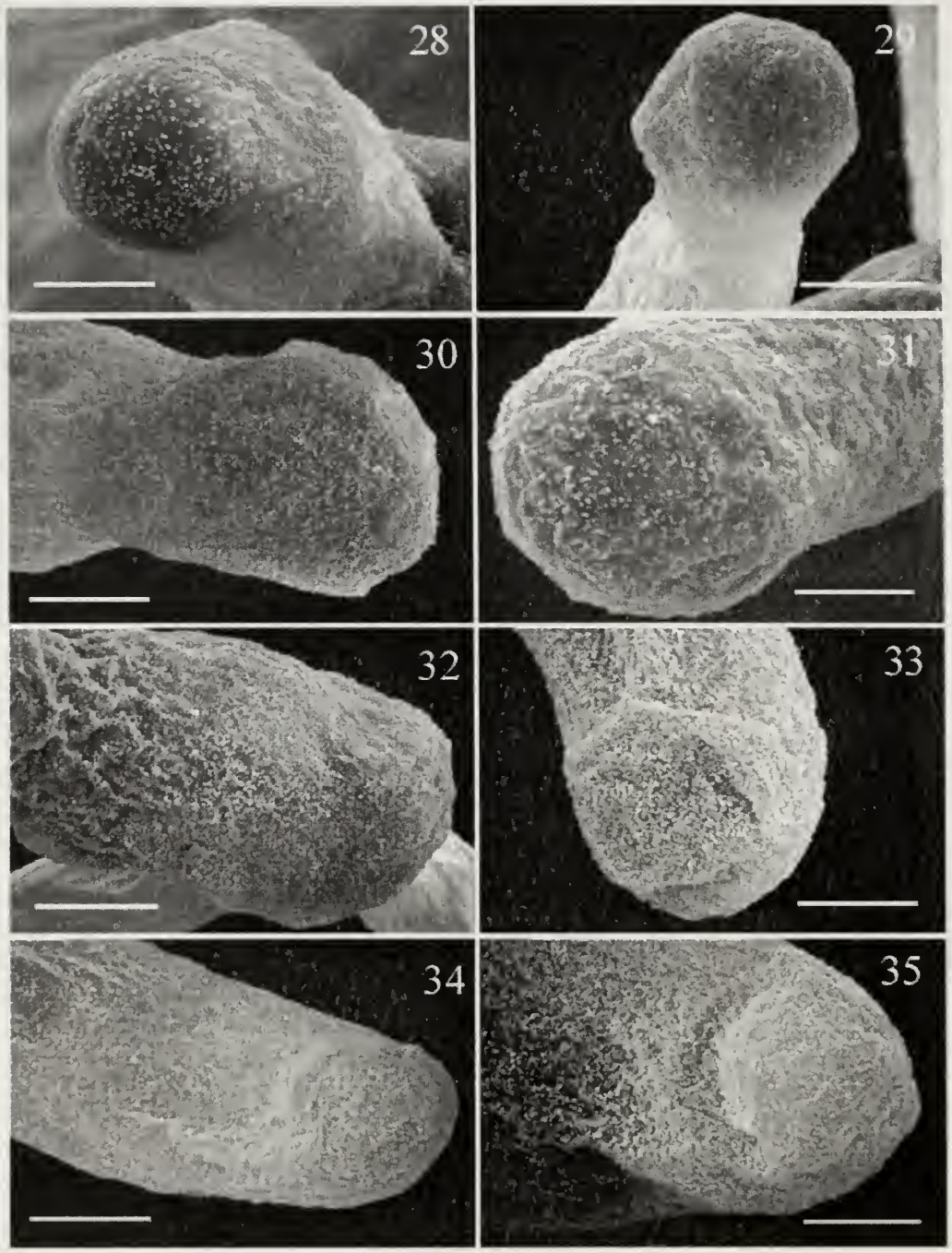
Figs. 4-11. Scanning electron micrographs of elongating hyphae of *Achlya bisexualis* showing cellulose surface labeling with cellulase-gold. Note the unlabeled apices. Bars: 4-9 = 2.00 µm; 10 = 2.31 µm; 11 = 3.00 µm.



Figs. 12-19. Scanning electron micrographs of elongating hyphae of *Achlya bisexualis* showing surface labeling of cellulose with cellulase-gold. Note the gradual decrease of labeling towards the apices. Bars: 12 = 3.33 µm; 13 = 2.73 µm; 14 = 1.67 µm; 15-18 = 2.00 µm; 19 = 3.31 µm.



Figs. 20-27. Scanning electron micrographs of surface of non-elongating hyphae present in growing colonies of *Achlya bisexualis* showing cellulose labeling with cellulase-gold. Note the labeled apices. Bars: 20-22 = 3.00 µm; 23, 24 = 2.00 µm; 25 = 1.50 µm; 26 = 2.31 µm; 27 = 3.33 µm.



Figs. 28-35. Scanning electron micrographs of non-elongating hyphae from non-growing colonies of *Achlya bisexualis*, incubated in glucose-only medium showing surface labeling of apices for cellulose with cellulase-gold. Note the labeled apices. Bars: 28 = 2.31 µm; 29 = 4.29 µm; 30 = 5.00 µm; 31 = 1.50 µm; 32, 33 = 3.75 µm; 34, 35 = 3.00 µm.

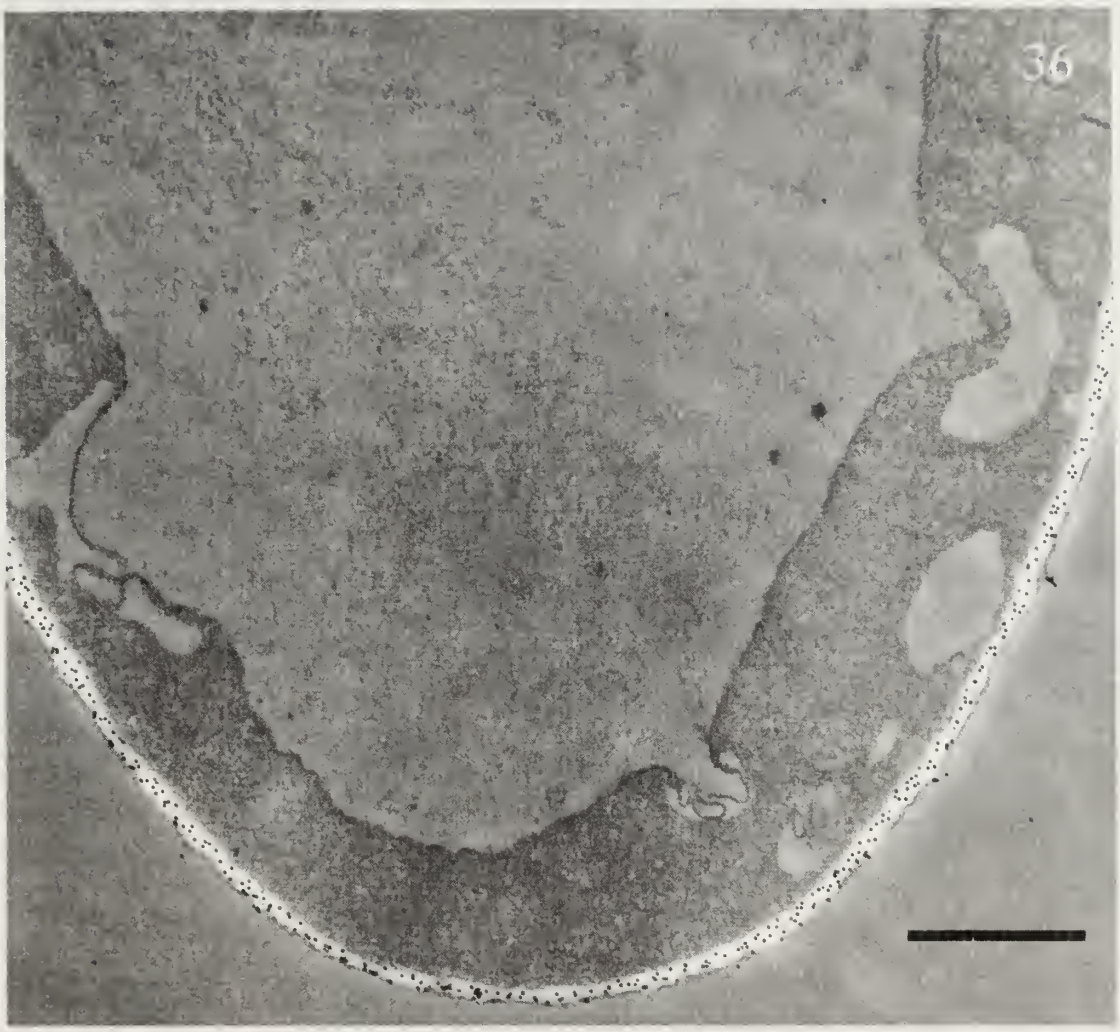


Fig. 36. Cross section of *Achlya bisexualis* hypha showing labeling of cellulose in the cell wall with cellulase-gold complex. Bar=1 µm.

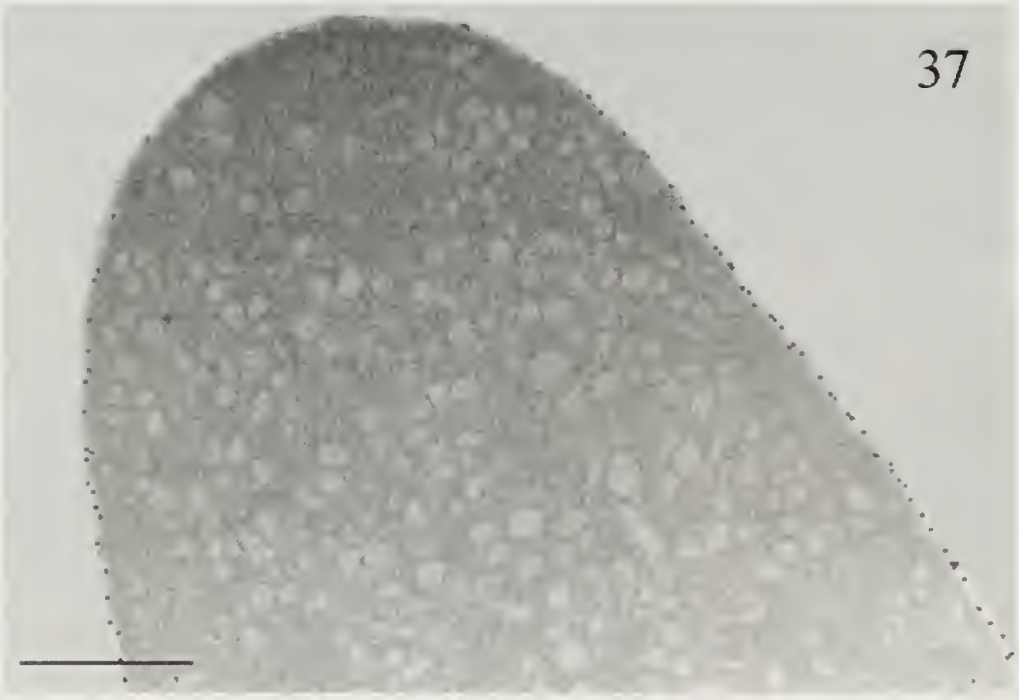


Fig. 37. Longitudinal section of the apical region of an elongating *Achlya bisexualis* hypha showing labeling of cellulose with cellulase-gold. Bar=1 µm.

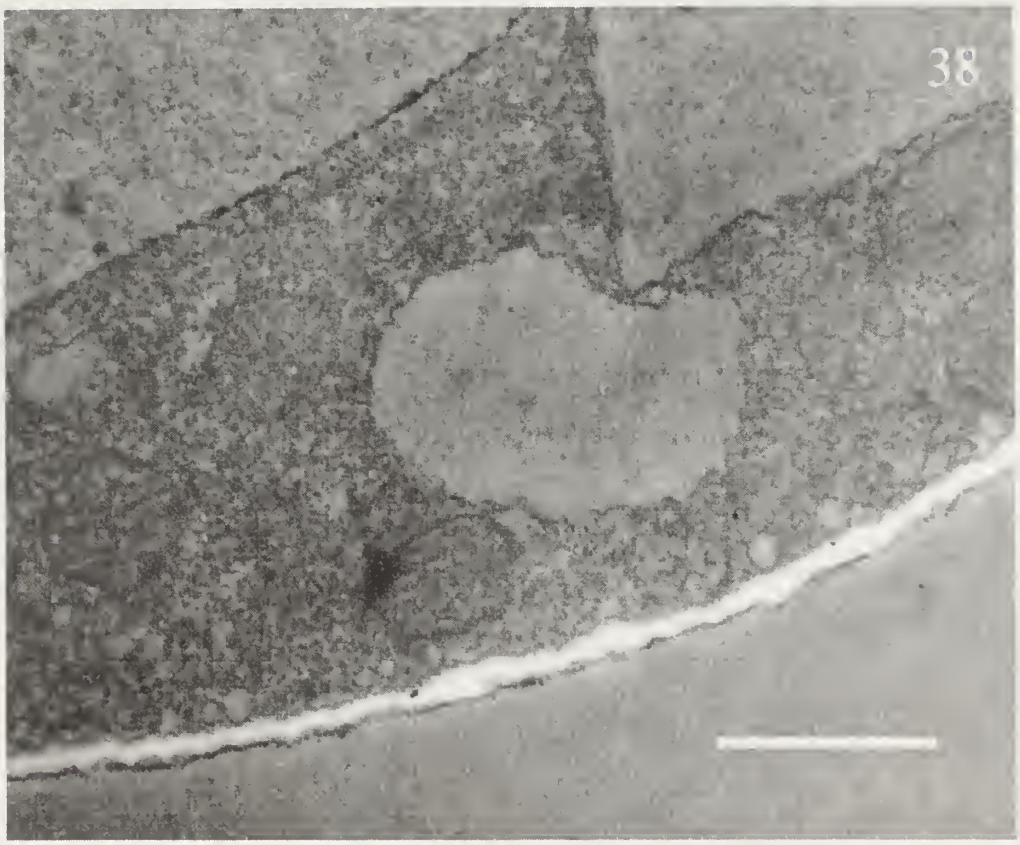
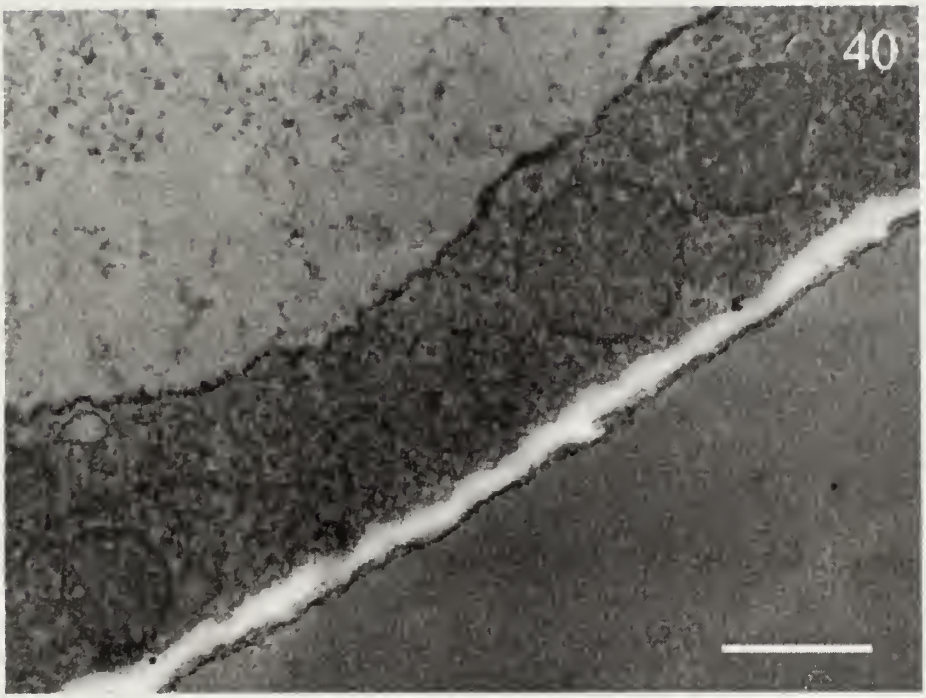
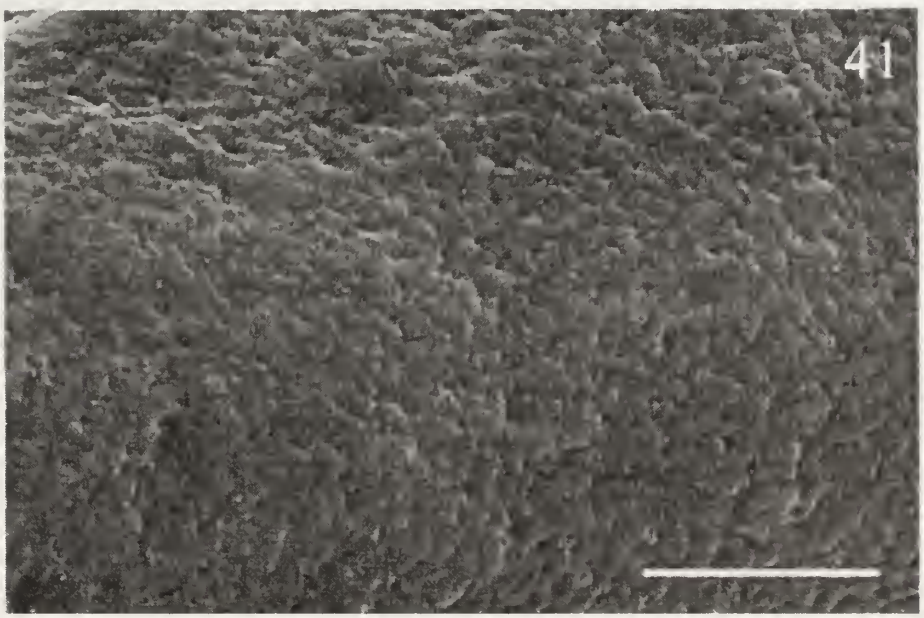


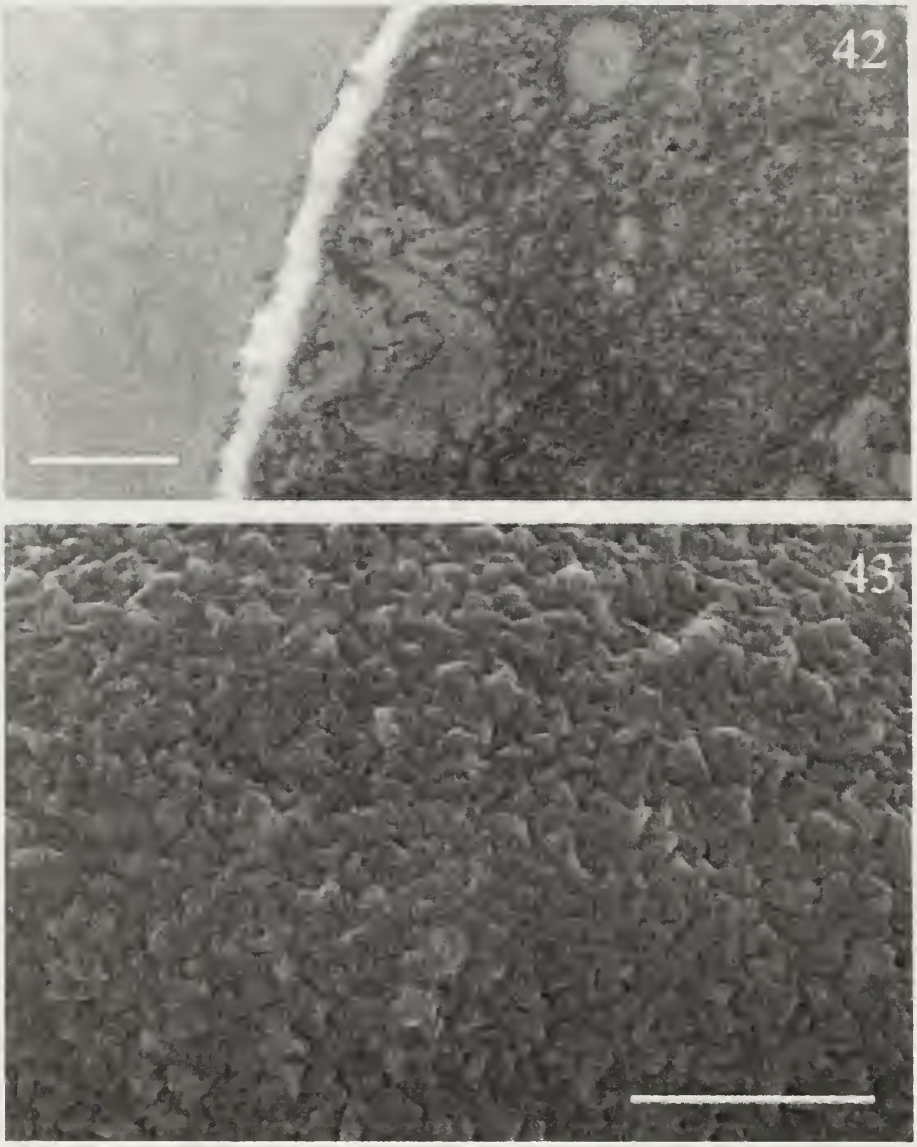
Fig. 38. Cross section of *Achlya bisexualis* hypha labeled for cellulose with endocellulase III-gold. Bar=0.5 µm.

Fig. 39. Negative staining of endocellulase III-gold complex. Bar=200 nm.

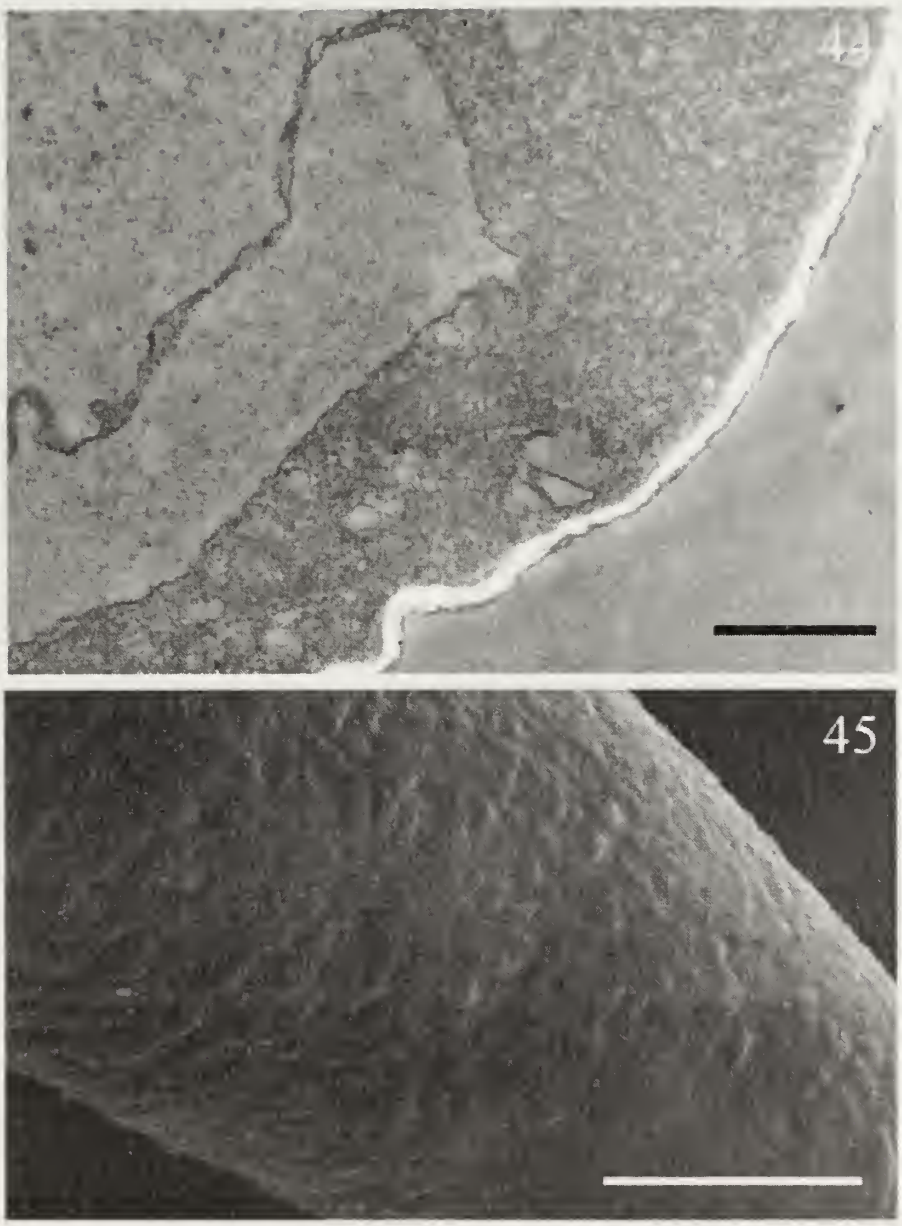




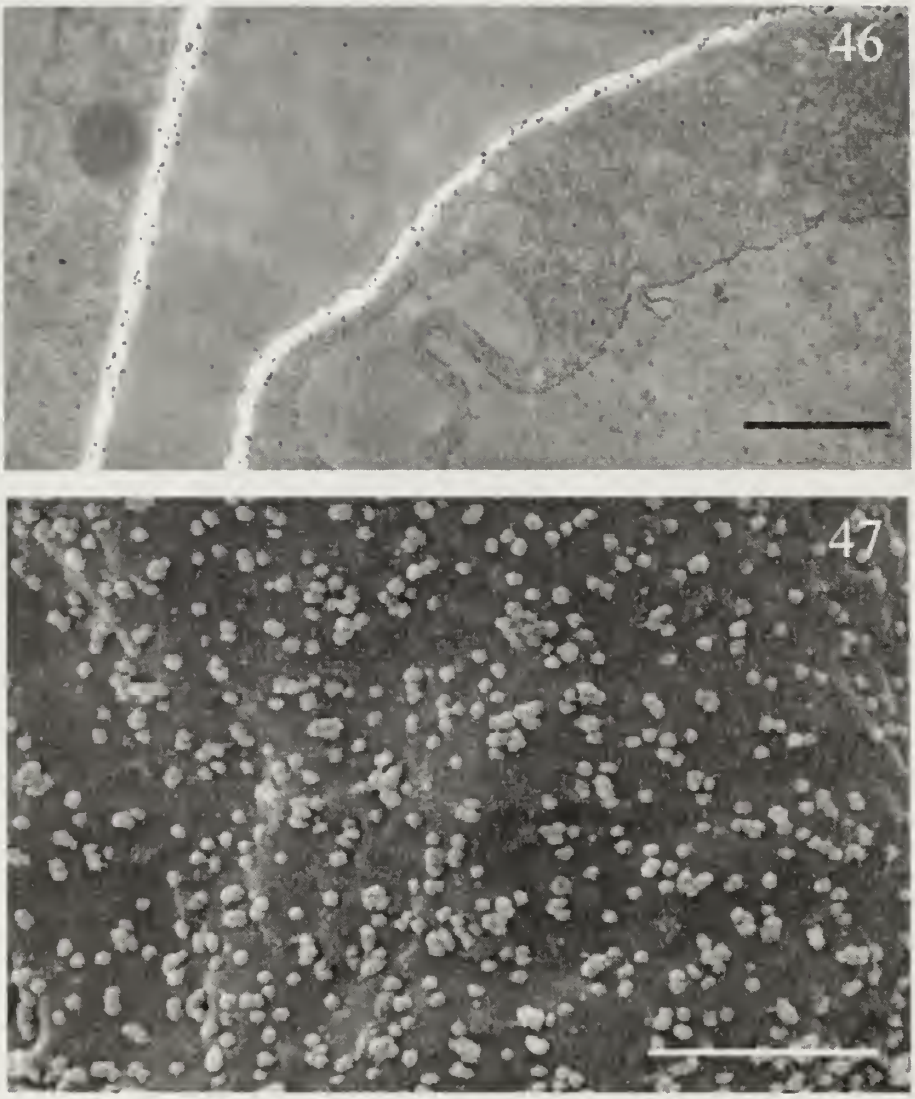
Figs. 40-41. Electron micrographs showing the absence of cellulose labeling with cellulase-gold in the cell wall of *Achlya bisexualis* resulting from the preabsorption of the labeling solution with CMC. Bars: 40=1 µm; 41=1.5 µm.



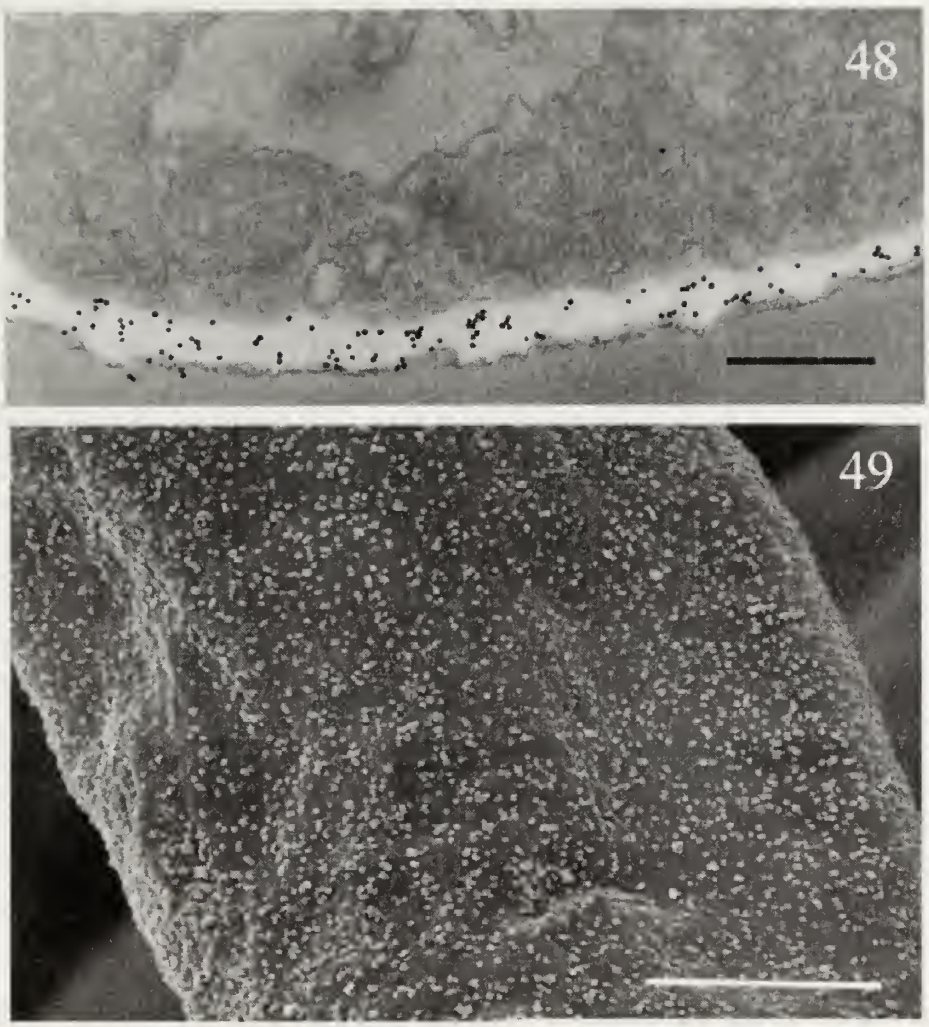
Figs. 42-43. Electron micrographs showing the absence of gold label in the cell wall of *Achlya bisexualis* resulting from the preincubation of the sections (TEM) or colonies (SEM) with colloidal gold-affinipure goat anti-mouse IgG. Bars: 42=1 µm; 43=1.5 µm.



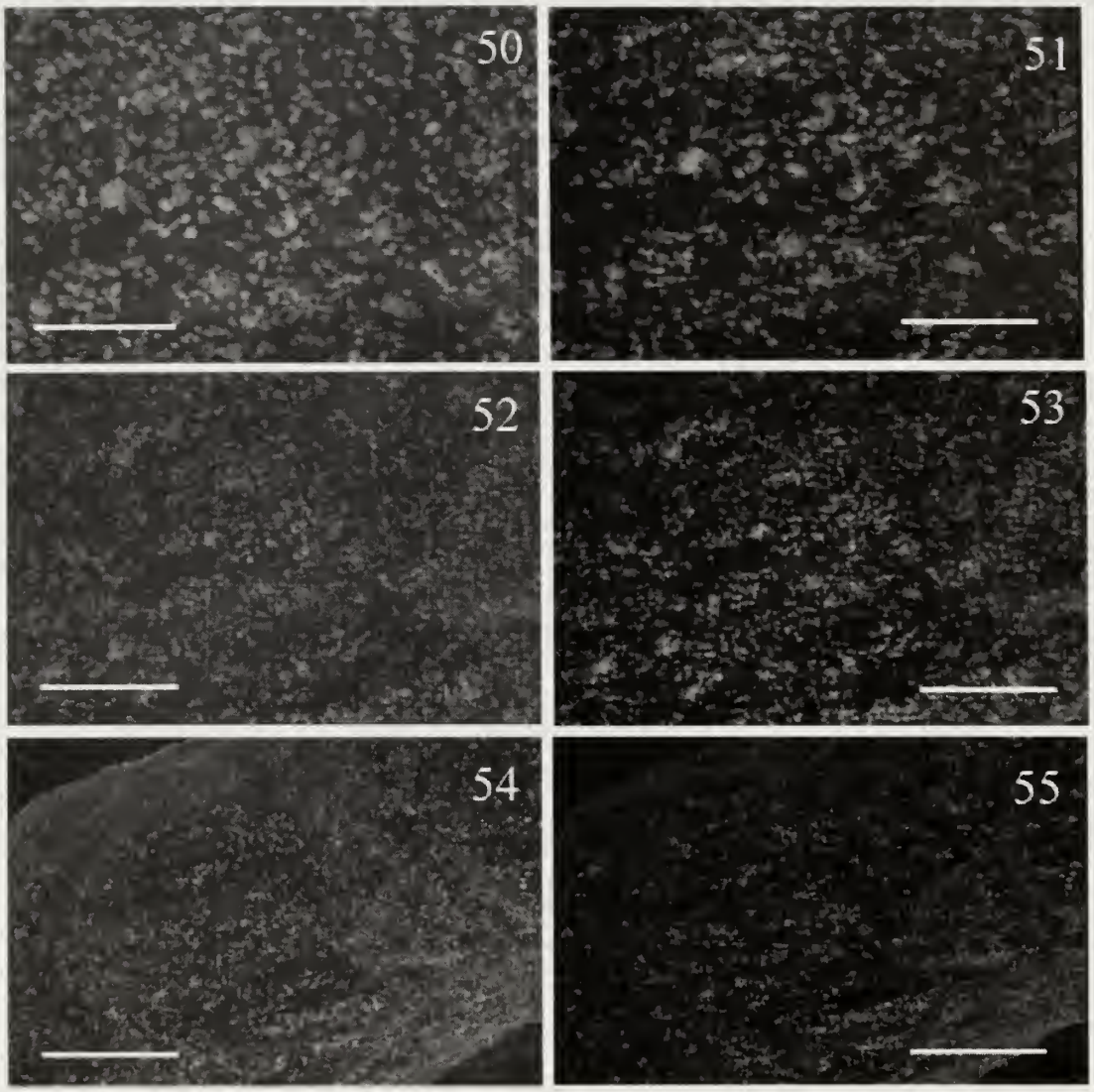
Figs. 44-45. Electron micrographs showing the absence of cellulose labeling with cellulase-gold in the cell wall of *Achlya bisexualis* resulting from the pretreatment of the sections (TEM) or colonies (SEM) with cellulase. Bars: 44=1 µm; 45=1.67 µm.



Figs. 46-47. Electron micrographs showing the regular pattern of cellulose labeling with cellulase-gold in the cell wall of *Achlya bisexualis* resulting from the preincubation of the labeling solution with chitozan. Bars: 46=1 µm; 47=1.2 µm.



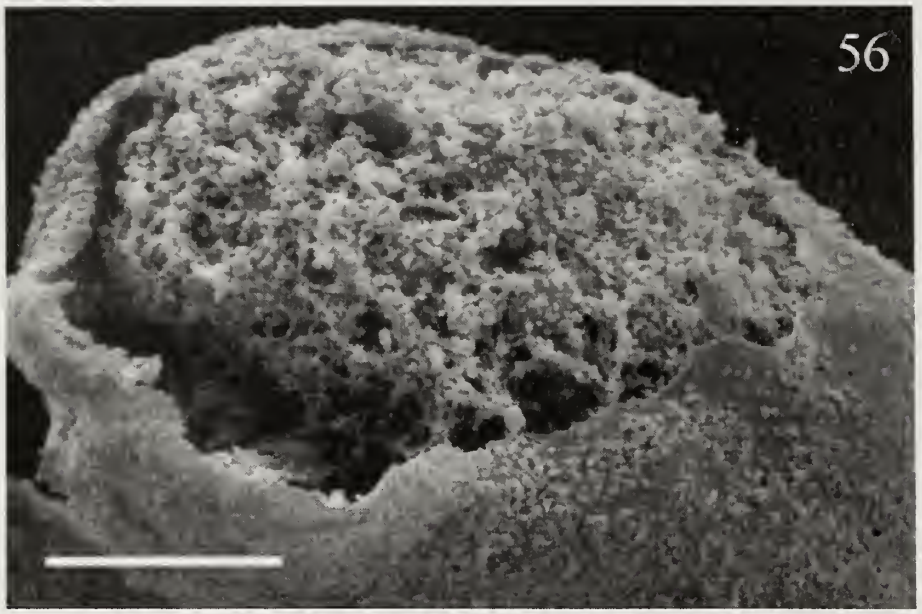
Figs. 48-49. Electron micrographs showing the regular pattern of cellulose labeling with cellulase-gold in the cell wall of *Achlya bisexualis* resulting from the preincubation of the labeling solution with laminarin. Bars: 48=0.5 µm; 49=3 µm.

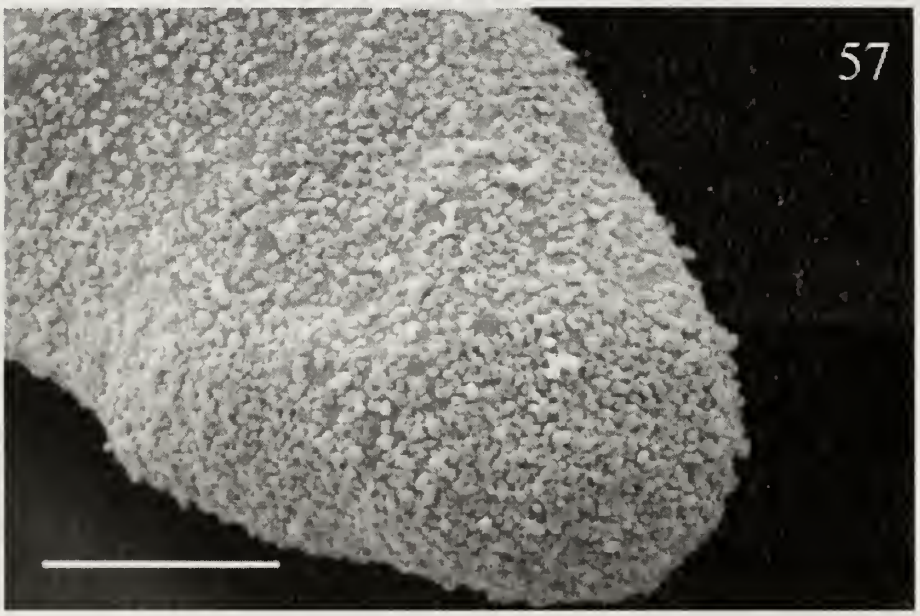


Figs. 50-55. Scanning electron micrographs showing the regular pattern of cellulose labeling with cellulase-gold on the hyphal surface of *Achlya bisexualis*. 50, 52, 54. Secondary images. 51, 53, 55. Backscatter images of the same regions. Bars: 50 and 51=1.5 µm; 52 and 53=3.00 µm; 53 and 55=857 nm.

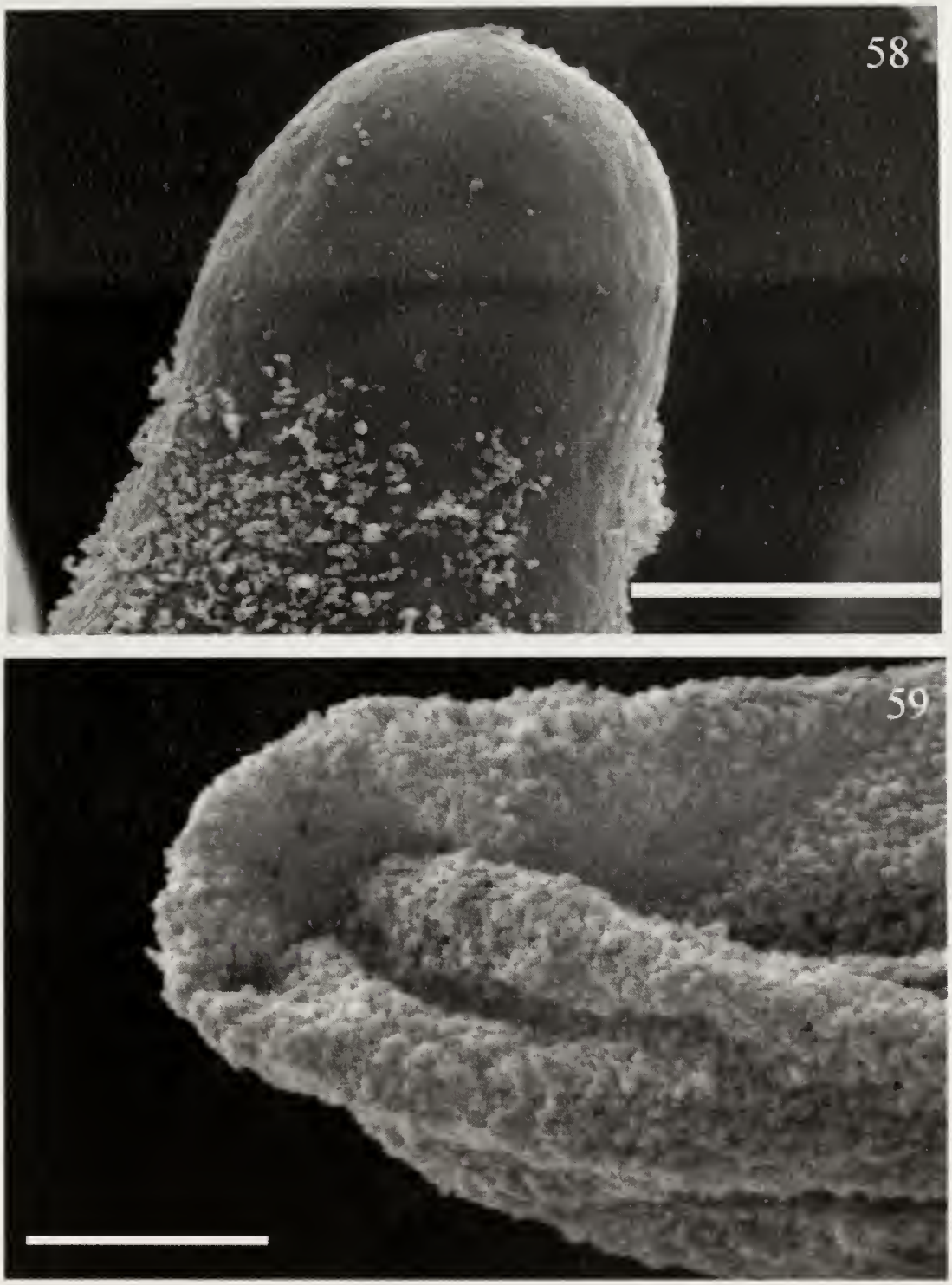
Table 1. Glucose equivalent from standard curve showing cellulase activity during labeling

Sample	Glucose equivalent from standard curve(mg/ml)	
	Reaction time: 30 min	Reaction time: 3 hrs
Whole wall and cellulase-gold	0.009	0.009
Whole wall and buffer	0.005	0.006
Small colony and cellulase-gold	0.004	0.004
Small colony and buffer	0.002	0.002
Cellulase-gold	0.006	0.007
CMC and cellulase-gold	0.02	0.04

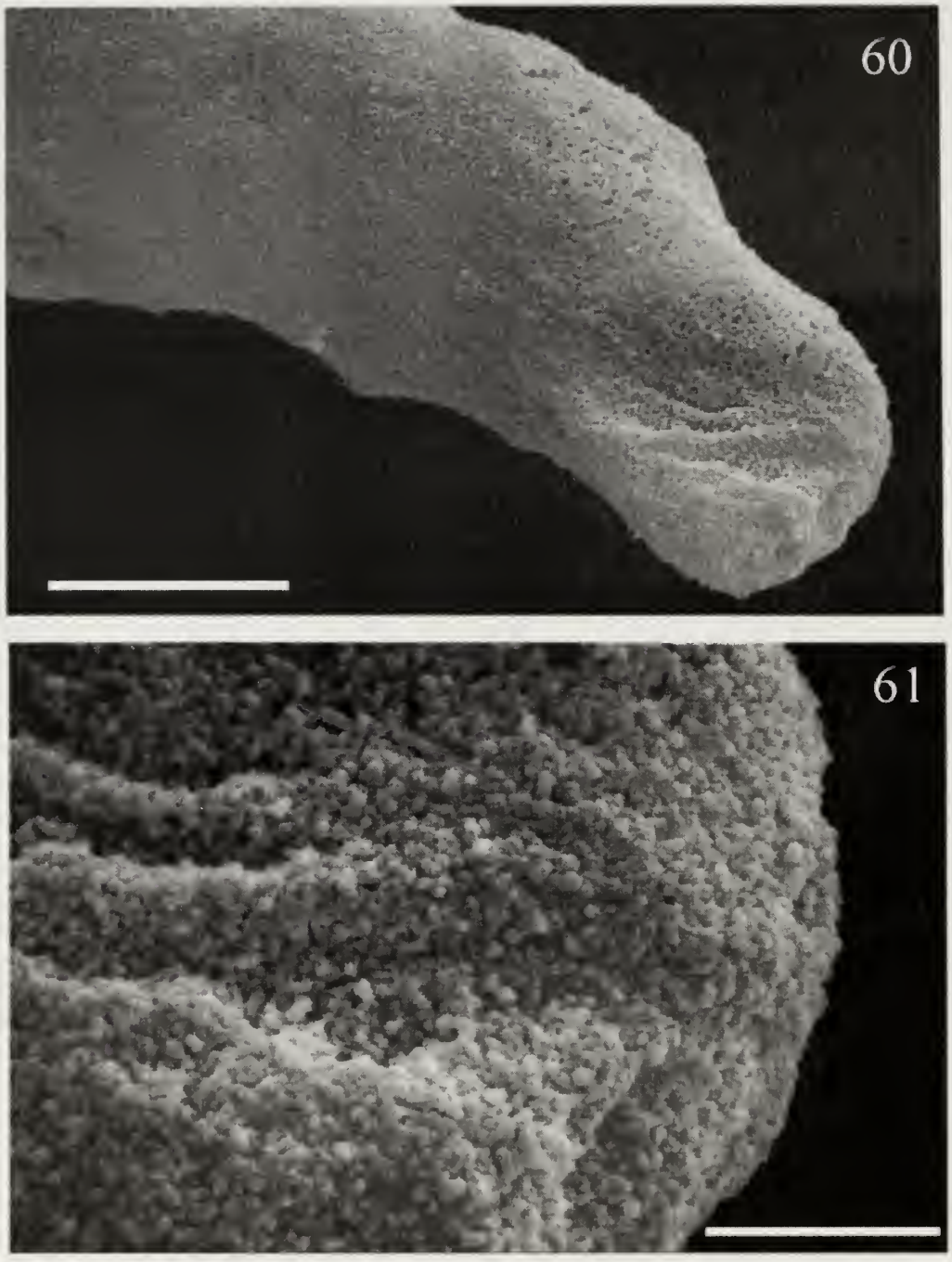




Figs. 56-57. Scanning electron micrographs showing cellulose labeling with cellulase-gold of elongating *Achlya bisexualis* hyphae that were hydrolyzed with zymolyase before chemical fixation. Bars: 56=2.31 µm; 57=2.00 µm.



Figs. 58-59. Scanning electron micrographs showing cellulose labeling with cellulase-gold of elongating *Achlya bisexualis* hyphae that were hydrolyzed with zymolyase after chemical fixation. Bars: 58=2.31 µm; 59=1.00 µm.



Figs. 60-61. Scanning electron micrographs showing cellulose labeling with cellulase-gold of an *Achlya bisexualis* hypha from a non-growing colony treated with zymolyase. Bars: 60=3.33 µm; 61=857 nm (higher magnification of the apex).

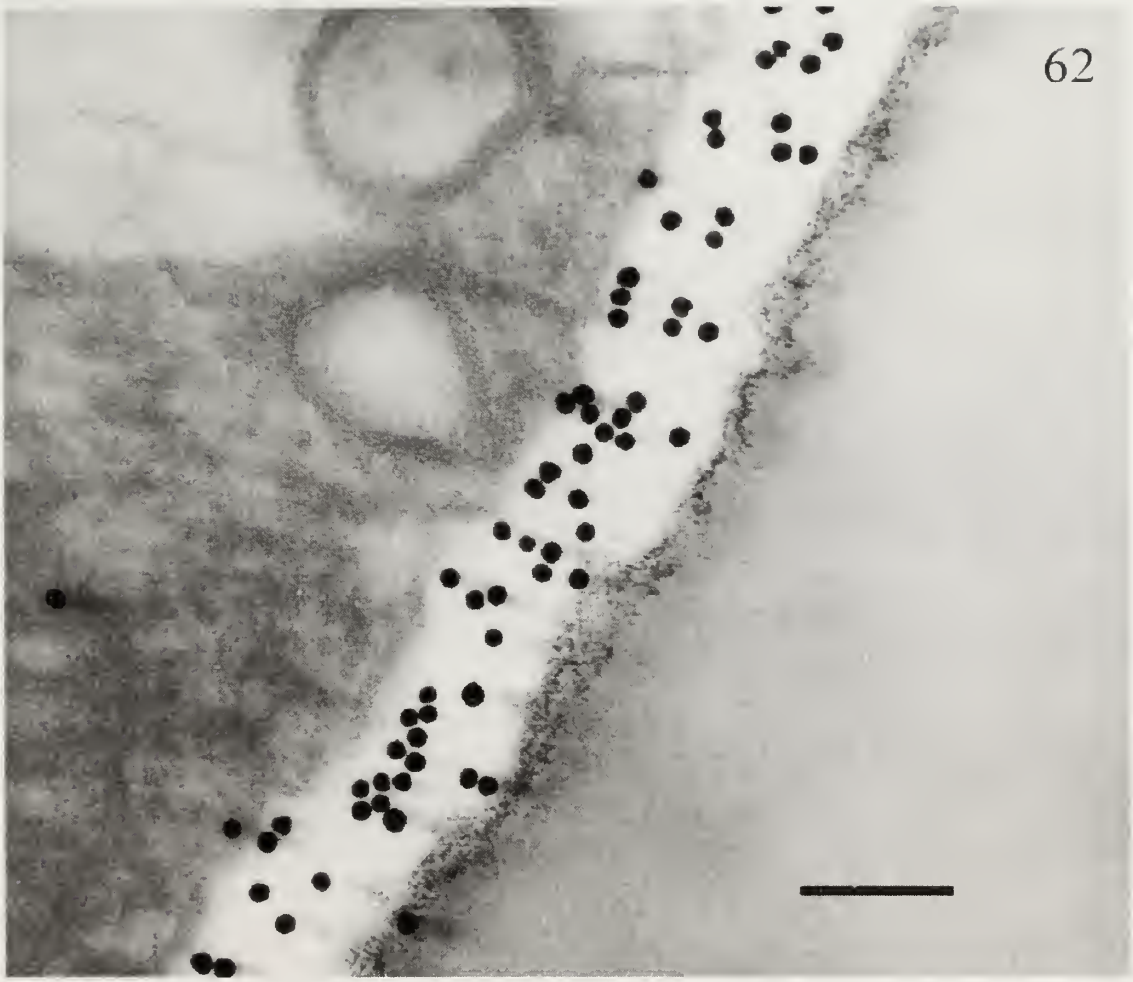


Fig. 62. Transmission electron micrograph of cross section of *Achlya bisexualis* hypha from a colony incubated in 100 µM DCB. Cell wall cellulose is labeled with cellulase-gold complex. Bar=0.5 µm.

is relatively higher when the enzyme-gold complex reacted with CMC.

Cellulose Localization on the Surface of the Hyphae in Colonies Incubated with Zymolyase

In the colonies, growing in PYG medium, that were hydrolyzed with zymolyase prior to fixation, most of the hyphal apices are intact and labeled. A small portion of the hyphae has broken apices. In these hyphae the remaining cell wall is labeled with cellulase-gold complex (Fig. 56, 57).

In the growing colonies that were chemically fixed first and then hydrolyzed with zymolyase, all the hyphae have intact apices. Most of the apices are labeled, but some are not (Fig. 58,59).

The non-growing colonies from glucose-only medium were screened for the absence of elongating hyphae prior to the treatment. Time of fixation, before or after hydrolysis, did not make any difference in the results. All the hyphae in these colonies are intact and are labeled as intensively as the rest of the hyphal regions (Fig. 60, 61).

Hyphal Elongation, Spore Germination and Cellulose Localization in the Presence of DCB

The presence of DCB in the medium does not affect the growth of *Achlya*. It does not affect the morphology of the hyphae or the growth rate. The average hyphal elongation rate is 3.6 µm/min. Spores germinate equally

well in PYG medium with and without DCB. There is no difference in the cellulose surface labeling of hyphae from the colonies treated with DCB and regularly growing colonies. The labeling patterns and their ratio is the same (data not shown). Cross sections of hyphae from the colonies treated with DCB have expected pattern of cellulose labeling (Fig. 62).

Discussion

The cellulase-gold affinity labeling is specific for cellulose in the fungus *Achlya*, based on its reproducibility and a large variety of controls. Differences in the fixation techniques do not affect the labeling pattern. Standard chemical fixation gave the same results as freeze-substitution and cold methanol fixations. The results are highly reproducible and are not artifacts of the fixation procedure. Thus this labeling technique provides a specific and reliable method for localizing cellulose on thin sections and hyphal surfaces.

The fact that cellulose labeling was found on the hyphal surface, may contradict a general assumption that the microfibrilar component of the cell wall, cellulose in the case of *Achlya*, is located next to the plasma membrane and is covered by the matrix components of the wall. The cell wall may not be arranged as layers of components, but as a mixture. This would explain the presence of some cellulose on the surface, this

explanation seems unlikely since *Achlya* secretes cellulase during growth (Thomas and Mullins 1967, 1969), and if cellulose was present on the surface, it could be hydrolyzed. Furthermore, when cellulase is applied exogenuously to the living *Achlya* cultures, it does not destroy the integrity of the hyphae, nor does it change their surface as revealed by shadow replicas (Reiskind and Mullins 1981b). The unique structure of the cellulase enzyme complex may explain the presence of cellulose labeling on hyphal surface. The cellulolytic enzyme complex from *Trichoderma reesei* used here for labeling consists of a number of enzymes: endoglucanases (EG); cellobiohydrolases (CBH); and cellobiase (CB); which work synergistically. All these enzymes contain a small highly homologous 36-residue region called the A domain, connected to the globular enzymatically active core domain by a threonine- and serine-rich sequence. The A domain has no catalytic activity in CBH I and CBH II, but it is thought to have a cellulose-binding function. The core protein alone does not have full cellulose-hydrolysing activity, but has normal activity on small synthetic substrates (Rouveinen et al. 1990). Perhaps cellulases are able to bind cellulose microfibrils located inside the wall via the small cellulose binding domains (CBD). CBD could penetrate the wall and find the binding sites, while the catalytic domains, conjugated to gold remain on the surface. The

results with EG III labeling indirectly prove the idea of CBD penetrating the wall and leaving the catalytic domains attached to gold on the surface. EG III provided by Dr. Tim Fowler (Genencor International, Inc.) is a genetically modified enzyme that does not have a cellulose binding domain (personal communication). Without the binding domain, this enzyme can not attach to the cellulose microfibrils and it results in the lack of EG III-gold affinity labeling.

The results of the experiments that measured cellulase-gold activity during labeling also provide a support for the idea of CBD penetrating the wall and leaving the catalytic domain attached to gold on the surface. The enzyme-gold does not show enzyme activity against a whole wall sample or a colony, but is active against the soluble cellulose derivative CMC. Perhaps, in the case of whole wall and colony treatments the cellulose binding domain finds the binding site by penetrating the wall and then attaches to cellulose. The catalytic domain conjugated to gold does not get access to cellulose microfibrils surrounded by the matrix material of the wall. Thus, the binding takes place without hydrolysis. In the case of CMC the cellulose microfibrils are not covered, they are available for the catalytic domain. Therefore, in this case both binding and hydrolysis take place.

According to the results of cellulase-gold labeling, all the hyphae from non-growing colonies (glucose-only medium) are evenly labeled in all regions, including the apices. Light microscope screening prior to the EM processing ensured that these hyphae are not elongating. On the other hand, cellulase-gold labeling of the hyphae from growing colonies (PYG medium) revealed three patterns of cellulase-gold labeling at the apices: labeled (small portion of hyphae), unlabeled and with the decreasing label towards the apex. Light microscopic observations prior to fixation revealed that a small portion of hyphae in these colonies is not elongating, but the majority of the hyphae are elongating. Based on this, I propose that in non-elongating hyphae cellulose is evenly distributed along the hypha and is present in the apex. Elongating hyphae lack cellulose at the apices or there is a gradual decrease in the amount of cellulose toward the apices.

The results of the experiments with zymolyase hydrolysis support the conclusion that in some hyphae in growing colonies there is no cellulose in the apical cell wall. In the colonies that were treated with zymolyase, prior to fixation, these hyphae have broken apices. I explain this by the fact that the wall in these apical regions lacks cellulose and consists mainly of 1,3-ß-glucans. Zymolyase has both 1,3-ß-glucanase and protease activities. It hydrolyzes not only

1,3-ß-glucans, but also structural wall proteins, cell membrane proteins and cytoplasmic proteins. Thus, the elongating regions have "hydrolyzed" apices. In the colonies that were chemically fixed first and then hydrolyzed with zymolyase, the elongating hyphae have intact unlabeled. The apices are not broken as in the previous case because chemical fixation crosslinks proteins so they can not be hydrolyzed. As expected, in both experiments, non-elongating hyphae (glucose-only medium) have intact apices with cellulose labeling as intensive as in the other regions of the hyphae.

The experiments with DCB gave an unexpected result. In these experiments it was the intention to use a different approach to show the absence of cellulose in the wall of elongation regions. DCB is a classic inhibitor of cellulose biosynthesis in higher plants (Delmer 1999). It was expected that the hyphae would continue to elongate by synthesizing 1,3-ß-glucans and producing large regions of apical wall made mainly of this component and that these hyphal regions lacking the structural support of cellulose might not have a tubular form. However, DCB had no inhibition effect on the growth process of *Achlya*. There were no changes in hyphal morphology as revealed by light microscopy, TEM or SEM observations. Hyphal elongation rates in colonies incubated with DCB were the same as in the regular growth medium. Cellulose labeling of the cross sections and the

hyphal surface revealed no difference between the hyphae grown in regular medium or the medium with DCB. Perhaps, the cellulose biosynthesis system of *Achlya* is different from that found in higher plants in the step that is affected by DCB, or DCB molecules may not be able to penetrate the *Achlya* wall. Similar results for the lack of an inhibitory effect of DCB were found in the cellular slime mold *Dictyostelium* (Blanton 1997, Blanton personal communication). Actually, none of the three major cellulose-synthesis inhibitors used in higher plants--DCB, isoxaben, and pthoxazolin--had an effect in this organism.

CHAPTER 5
LOCALIZATION OF 1,3-ß-GLUCANS IN THE CELL WALL AS REVEALED BY ELECTRON MICROSCOPY AND CYTOCHEMICAL TECHNIQUES

Introduction

The term glucan applies to polysaccharides composed of glucose units and they are divided into alpha- and ß-anomers according to their stereochemistry around the anomeric carbon. The ß-glucans include both homopolysaccharides and heteropolysaccharides. Six different types of ß-glucans have been described in fungi: linear 1,3-glucans; 1,3-glucans with occasional 1-6 single glucose branches, with or without phosphate; 1,3-glucans with significant amounts of 1,6-branches; glucans containing mostly 1,6-linkages; glucans containing 1,3-, 1,4- and 1,6- linkages (Ruiz-Herrera 1991).

These ß-glucans are getting attention because of their potential application in chemical, pharmaceutical and food industries. Pharmaceutically, 1,3-ß-glucans that have ß-glucopyranosyl units attached by 1->6 linkages as single unit branches have been shown to enhance the immune system. This enhancement results in antitumor, antibacterial, antiviral, anticoagulatory and wound healing activities (Bohn and Bemiller 1995).

The 1,3-ß-glucans are important components of fungal cell walls and they are also storage carbohydrates in some fungi, especially in the Oomycetes and Basidiomycota. Some ß-glucans are secreted in the form of slimy material, and may protect cells from desiccation and other harmful environmental conditions (Ruiz-Herrera 1991). In the case of pathogenic fungi, ß-glucans are important in cellular recognition, and in eliciting defense responses of infected plants (Ryan 1987, Dixon and Lamb 1990, Cote and Hahn 1994).

Storage glucans accumulate intracellularly and are used as reserve material at critical stages of growth and reproductive development (Wang and Bartnicki-Garcia 1980, Lee and Mullins 1994). In *Phytophthora*, Wang and Bartnicki-Garcia (1973) reported a phosphorylated 1,3-ß-glucan in sporangia, zoospores and cysts. This phophorylated 1,3-ß-glucan contains one or two phosphate residues as monoester linkages at the C-6 hydroxyl groups of some glucose units. In *Achlya*, a phosphorylated cytoplasmic 1,3-ß-glucan has been isolated and characterized (Lee and Mullins 1994, Lee et al 1996), containing 5% phosphate (w/w), and has both mono-and diphosphoester linkages. The diester linkages are used to form very large polymers from the smaller neutral forms. Although the biological role of the reserve 1,3-ß-glucans is most often suggested as a source of energy or carbon

or both, in *Achlya* it is also an important site of phosphate storage (Lee and Mullins 1994).

The most general role of ß-glucans is a structural one, as the major component of fungal cell walls. Inhibition of ß-glucan synthesis in yeast leads to cell lysis and often death, resulting from a weakening of the cell wall (Perez et al. 1983, Miyata et al. 1985). Such inhibitors are used as important antifungal compounds against both plant and animal pathogens.

The 1,3-ß-glucans were localized on cross sections of *Achlya* (Shapiro and Mullins 1997). The method used indirect immunolabeling with a commercial polyclonal antibody specific for 1,3-ß-D-glucopyranose linkages. The glucans occurred in the cell wall, the large vesicles in the organelle-rich areas of the hypha, and in the large central vacuole in more mature areas. Preabsorption of the antibody with either purified neutral or phosphoglucan from *Achlya* completely eliminated subsequent labeling of hyphal sections. No labeling of the large population of apical vesicles was found, suggesting that these reserve glucans are not directly involved in apical growth. Since the labeling occurred in large vesicles and the central vacuole and no other cytoplasmic sites showed conjugation, the vesicle and vacuole membranes probably contain the synthases responsible for the biosynthesis of the reserve glucans. The labeling of serial sections revealed the

1,3-ß-glucans in both mature and apical regions. Additional labeling experiments have now been carried out on hyphae that were first determined to be elongating, as described in Chapter 3, to ensure that elongating apices contained 1,3-ß-glucans. Recall that in Chapter 4, evidence was presented that clearly demonstrated a lack of cellulose in elongating apices.

Materials and Methods

Culture Methods, Fixation and Microscopy Techniques

The general culture methods, fixation and microscopy techniques are described in Chapter 3 and Chapter 4.

Localization of 1,3-ß-Glucans Using Monoclonal Antibody

The primary antibody, raised in mouse against a laminarin-haemocyanin conjugate, was purchased from Biosupplies Australia PtyLtd (Parkville Victoria, Australia), catalogue number 400-2. This antibody recognizes linear 1,3-ß-oligosaccharide segments in 1,3-ß-glucans. The epitope includes at least five 1,3-ß-linked glucopyranose residues. It has no cross reactivity with 1,4-ß-glucans or 1,3-ß-, 1,4-ß-glucans (Meikle et al. 1991). It was diluted 1:100 in phosphate buffered saline (PBS), pH 7.2 containing 0.5 % cold water fish gelatin. The gold reagent, 18 nm Colloidal Gold-Affinipure Goat Anti-Mouse IgG (H+L) was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, Pennsylvania), catalogue number 115-215-146. The nickel grids with sections were floated on PBS containing

1% gelatin for 30 min to block non-specific labeling. Then the grids were floated on the top of 20 µl drops of the primary antibody solution for 1 h. After three washes in PBS the grids were floated on a 1:20 dilution of the gold reagent in PBS for 1 h. The solution was centrifuged at 18 000 *g* in a microcentrifuge for 1 min before use. Finally, the grids were washed: twice in PBS and twice in water.

The colonies destined for SEM observations were treated with the same series of solutions while completely submerged into them, rather than floated.

Cytochemical Controls

(1) Preabsorbtion of primary antibody with laminarin: in order to determine that the primary antibody binds to 1,3-ß-linkages it was preabsorbed with laminarin. Laminarin from *Laminaria digitata* was purchased from Sigma (St. Louis, Missouri), catalogue number L-9634. The grids with sections were blocked against nonspecific labeling as described above. Primary antibody stock solution, 10 µl, was incubated with 100 mg of laminarin in 1 ml of PBS containing 0.5 % gelatin for 1 h. The grids were floated on a drop of this solution for 1 h. The labeling procedure as described above was then followed. (2) Omission of the primary antibody: the procedure was the same as during the regular labeling, but the incubation with the primary antibody was omitted. (3) Replacement of the primary antibody with a non-

specific primary antibody raised in mouse: the procedure was the same as during the regular labeling but the primary antibody was replaced by an undiluted non-specific antibody (HL 1099) raised against neurofilaments in mouse. HL 1099 was provided by the Hybridoma Laboratory, Interdisciplinary Center for Biotechnology Research (Gainesville, Florida).

Results

Localization of 1,3-ß-Glucans on Sections and Hyphal Surfaces Using Monoclonal Antibodies

On cross sections of *Achlya* the monoclonal antibody detected 1,3-ß-glucans in the wall, small vacuoles, and the large central vacuole of mature regions (Figs. 63, 64). No cytoplasm-specific labeling was found. Longitudinal sections of both elongating (Fig. 65) and non-elongating (data not shown) hyphae show antibody labeling in the cell wall of the apices and all along the hyphae. No surface labeling was found using SEM (Fig. 66).

Cytochemical Controls

(1) Preabsorbtion of primary antibody with laminarin resulted in the absence of labeling (Fig. 67). (2) Omission of the primary antibody resulted in the absence of the labeling (Fig. 68). (3) Replacement of the primary antibody with a non-specific primary antibody raised in mouse resulted in the absence of the labeling (Fig. 69).

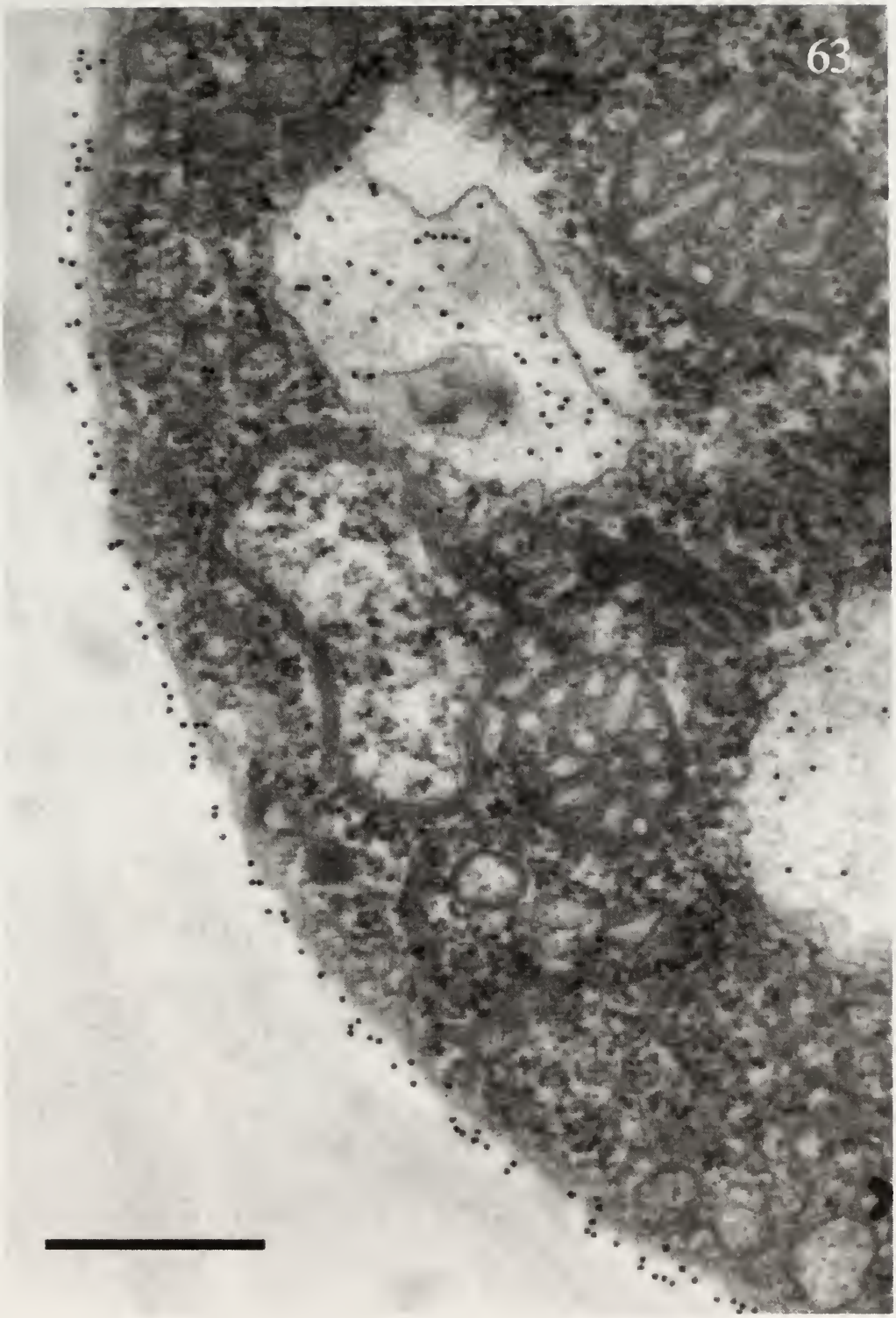


Fig. 63. Transmission electron micrograph showing localization of 1,3-ß-glucans with monoclonal antibody on cross section of *Achlya bisexualis* hypha. Bar=0.5 µm.

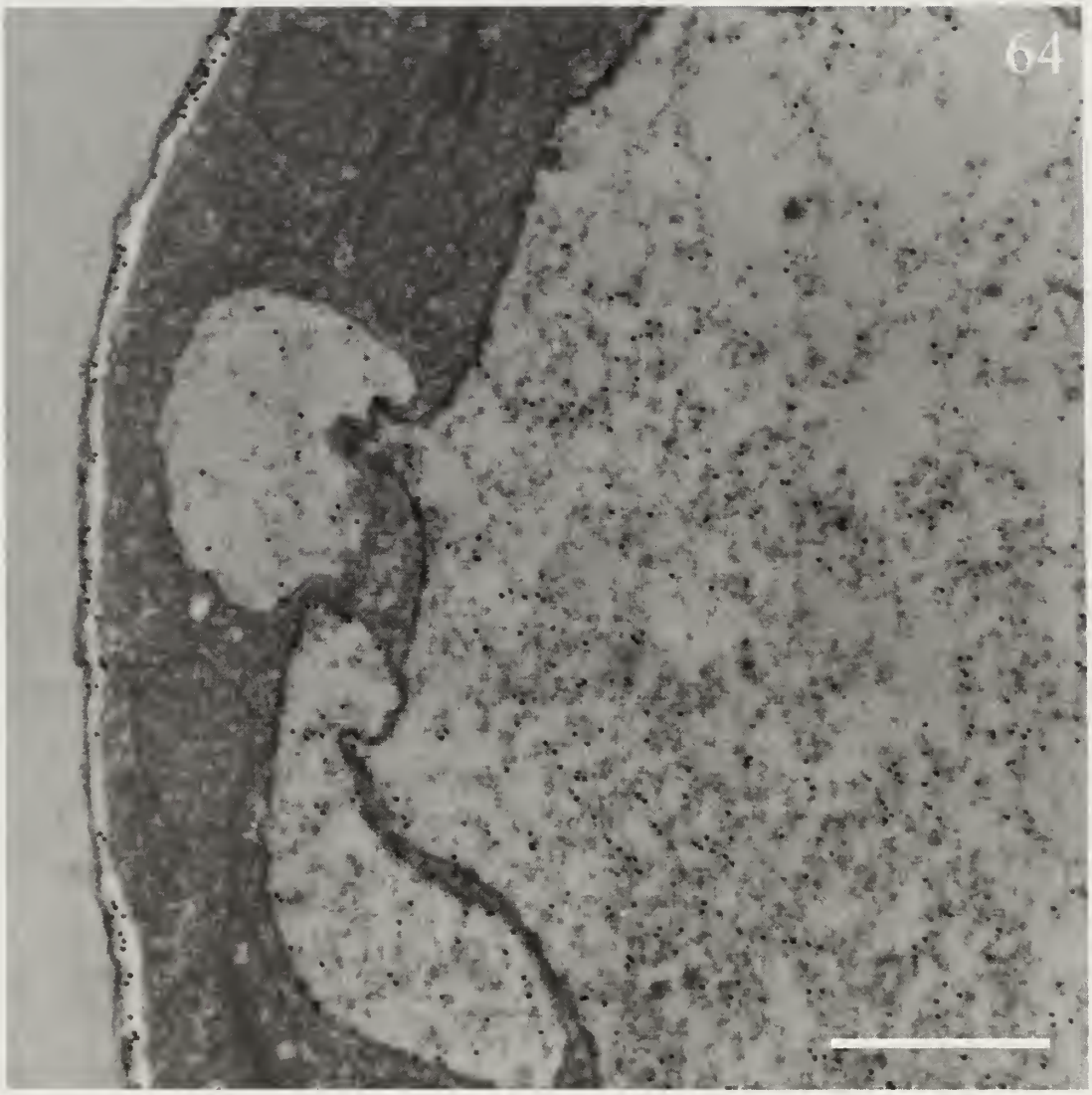


Fig. 64. Transmission electron micrograph showing localization of 1,3-ß-glucans with monoclonal antibody on cross section of *Achlya bisexualis* hypha. Bar=1.00 µm.

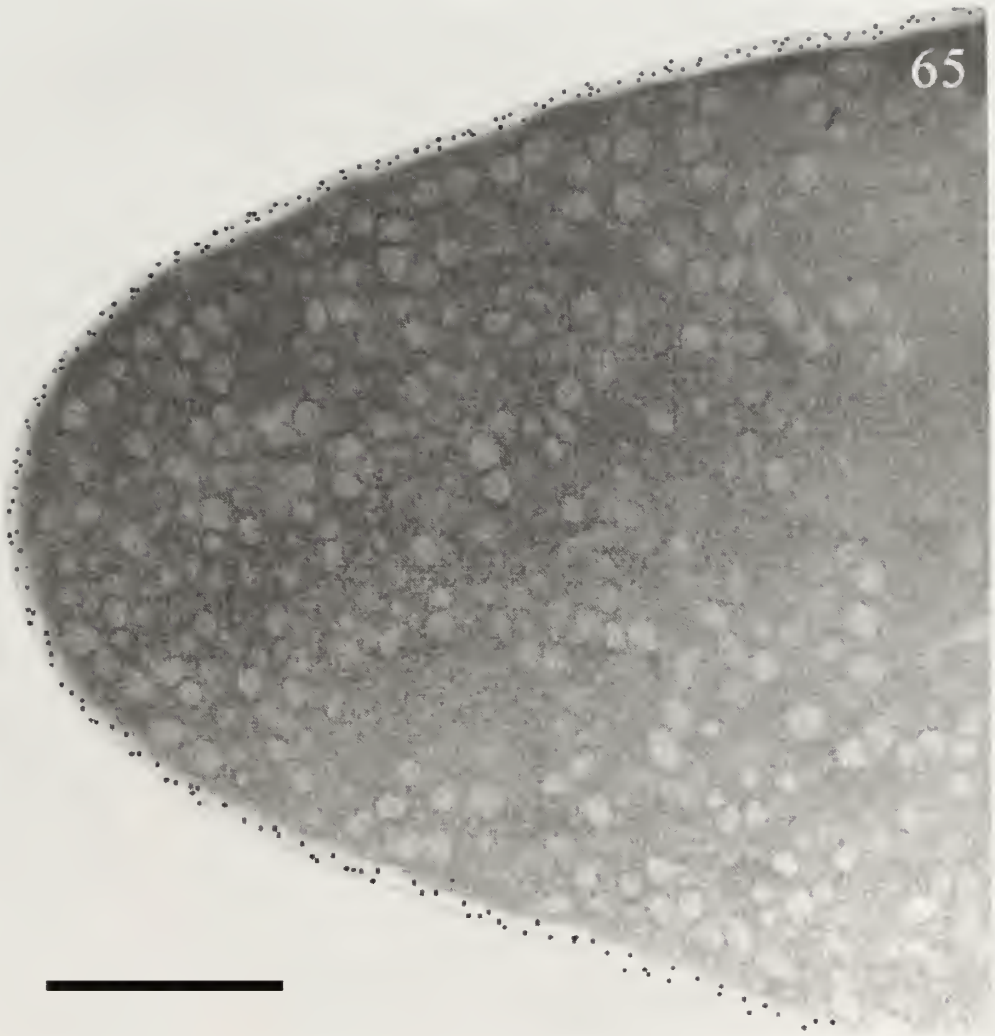


Fig. 65. Transmission electron micrograph showing localization of 1,3-ß-glucans with monoclonal antibody on longitudinal section of an elongating *Achlya bisexualis* hypha. Bar=1.00 µm.

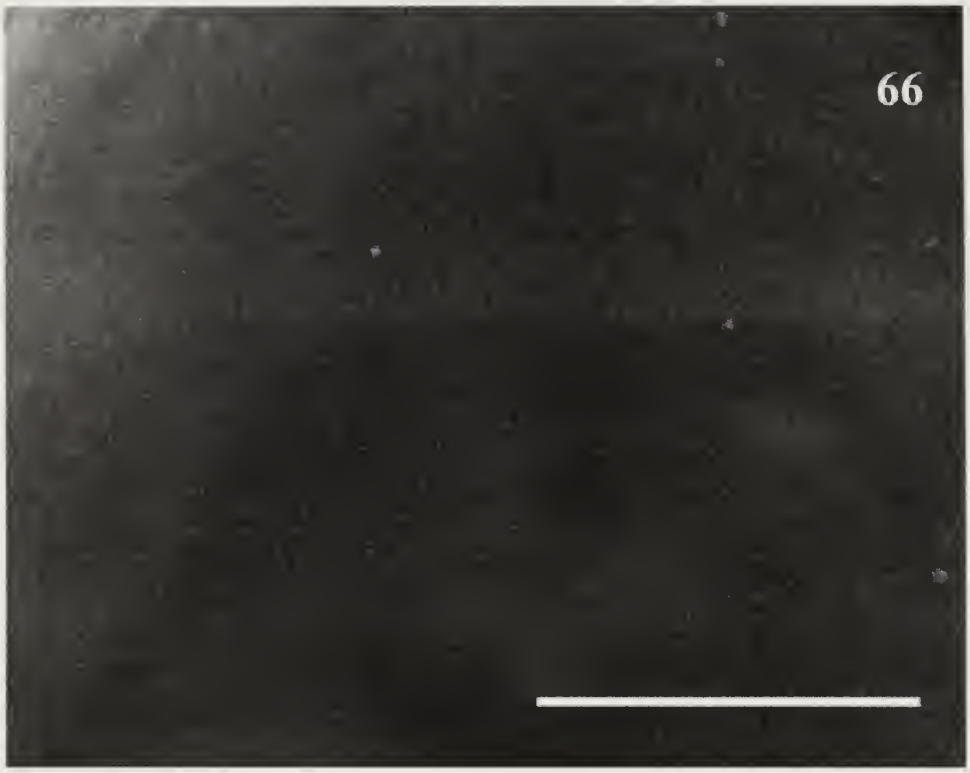
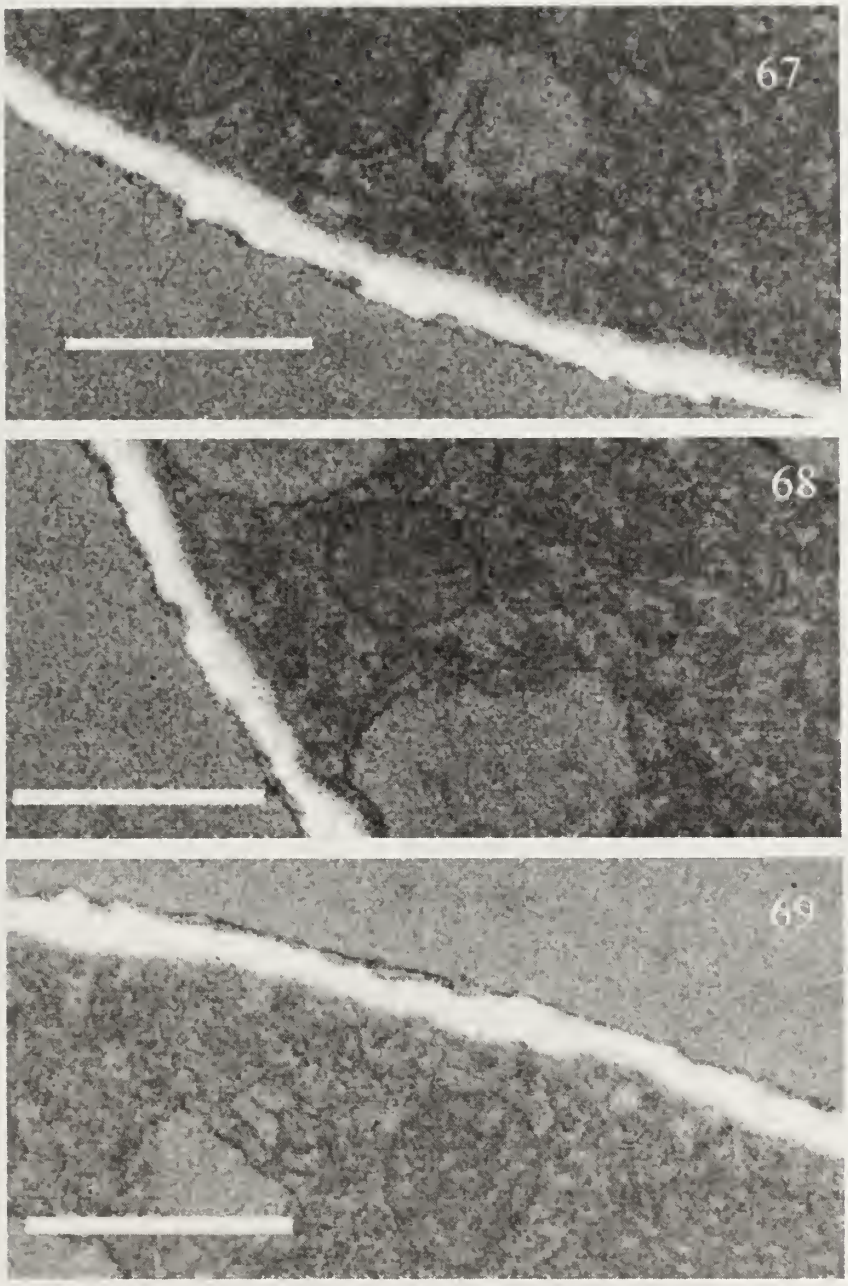


Fig. 66. Scanning electron micrograph showing the absence of 1,3-ß-glucans labeling on the surface of *Achlya bisexualis* hypha. Bar=2.31 µm.



Figs. 67-69. Transmission electron micrograph showing the absence of 1,3-ß-glucans labeling on the cross sections of *Achlya bisexualis* hyphae resulting from: 67. preabsorbtion of the labeling solution with laminarin. Bar=1.00 µm; 68. omission of the primary antibody. Bar=1.00 µm; 69. replacement of the primary antibody with a non-specific antibody raised in mouse. Bar=1.00 µm.

Discussion

Based on the results of the cytochemical controls, the monoclonal antibody used in the labeling procedure is specific for 1,3-ß-glucans. Previous results of labeling with a polyclonal antibody (Shapiro and Mullins 1997) are identical to the results of monoclonal antibody labeling. Both antibodies detect 1,3-ß-glucans in the cell wall and vacuoles. Previously, serial of cross sections of apical, subapical, and mature regions of a hypha were immunostained with the polyclonal antibody and strong labeling was found in the wall on all the sections (Shapiro 1995). Based on the data presented in Chapter 3, it can not be ascertained whether this hypha was elongating or non-elongating. In the present study, however, the elongating hyphae are distinguished from non-elongating ones and the distribution of 1,3-ß-glucans is compared. It is now possible to state that 1,3-ß-glucans are found in the apical wall of both elongating and non-elongating hyphae.

CHAPTER 6
LOCALIZATION OF CHITIN IN THE CELL WALL AS REVEALED BY ELECTRON MICROSCOPY AND CYTOCHEMICAL TECHNIQUES

Introduction

Chitin is the most characteristic polysaccharide of the fungal cell walls. It is an unbranched polysaccharide made of N-acetylglucosamine (GlcNAc) joined through 1,4-ß bonds. It was once thought to be absent in fungi containing cellulose, but a number of examples from all orders of the Oomycetes have demonstrated a least traces of chitin (Dietrich 1973, 1975). An insoluble fraction from the hyphal wall of *Achlya radiosa* Maurizio was characterized by x-ray and infrared analyses as chitin, and represented about 4% of the total wall (Campos-Takaki et al. 1982). The role of chitin in Oomycete cell wall remains unclear, and it has been suggested in *Saprolegnia* that chitin does not play an important role in morphogenesis based on results using the chitin synthase inhibitor polyoxin D (Bulone et al. 1992). An insoluble residue representing about 3% of the wall and containing glucosamine was reported in *Achlya* (Reiskind and Mullins 1981a). This fraction was identified as chitin by x-ray analysis (Mullins et al. 1984), but had unusual properties in that it is more highly crystalline than the

alpha-chitin normally observed in fungi and the characteristic lattice spacing was not readily perceptible. Thus chitin is clearly present in those fungi having cellulose as the major microfibrillar component; but its role is yet to be determined.

Materials and Methods

Chitin Localization Using Lectin

Tomato (*Lycopersicon esculentum*) lectin conjugated to gold was purchased from EY Laboratories, Inc. (San Mateo, California). The tomato lectin is described by the manufacturer as being specific for oligomers of 1,4-ß-linked N-acetylglucosamine, with the binding site being able to accommodate up to 4 carbohydrate units and these units do not have to be consecutive. The sections, on grids, were pretreated with phosphate buffer saline (PBS) containing 1% bovine serum albumin (BSA) at room temperature for 30 minutes. Then they were floated on the labeling solution for 30 minutes. The lectin-gold complex was a 1:9 dilution of the stock solution in PBS. The samples then were washed with PBS three times and rinsed twice in distilled water.

Cytochemical Control

To determine that the lectin-gold complex was binding to chitin, the probe was pre-incubated with re-acetylated glycol chitosan provided by Dr Michael N. Horst (Mercer University, Macon, Georgia). The glycol chitosan stock (0.118 g/100 mL) was diluted with PBS

(1:9). One part of the lectin-gold stock solution was diluted with nine parts of glycol chitosan and incubated for 30 min before labeling of the sections.

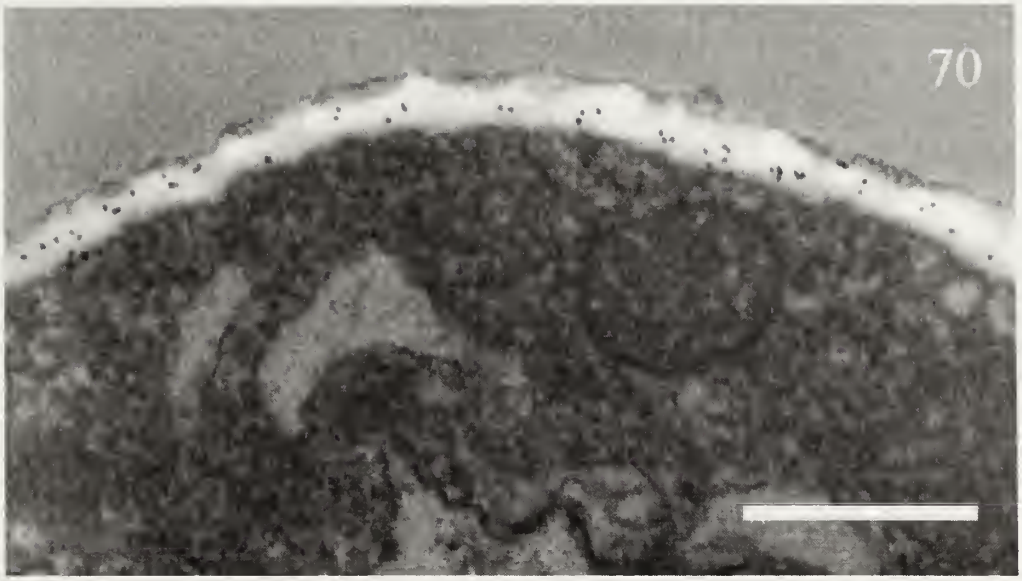
Results

Chitin is localized to the cell wall of *Achlya bisexualis* with tomato lectin-gold conjugate, where it is evenly distributed in the cross sections (Fig. 70). The chitin labeling is absent when the labeling solution is preincubated with glycol chitozan (Fig. 71).

Discussion

The tomato-lectin-gold conjugate appears to be a specific label for chitin in the cell walls of *Achlya*, based on the lack of labeling when the conjugate was pre-incubated with re-acetylated glycol chitosan. Previous studies on chitin (Campos-Takaki et al 1982, Mullins et al 1984, Gay et al 1992) demonstrated its presence in the cell walls of oomycetes with biochemical and biophysical analyses. This is the first report of the cytochemical localization and distribution of chitin in the walls of this group. Bulone et al 1992 described chitin as small globular particles in *Saprolegnia,* and found that hyphal growth and morphology were not altered when chitin synthesis was inhibited by polyoxin D. They concluded that chitin did not seem to play an important role in morphogenesis. Additional biophysical work on *Saprolegnia* (Gay et al 1992) describe chitin as localized small round granules of crystalline microfibrillar alpha

chitin. Chitin, however, synthesized in vitro appeared as spindle-like particles, and was not a skeletal polysaccharide involved in wall architecture. In regenerating protoplast walls it might have a secondary role in wall architecture, since it is microfibrillar. Thus the full role of chitin is still to be determined.



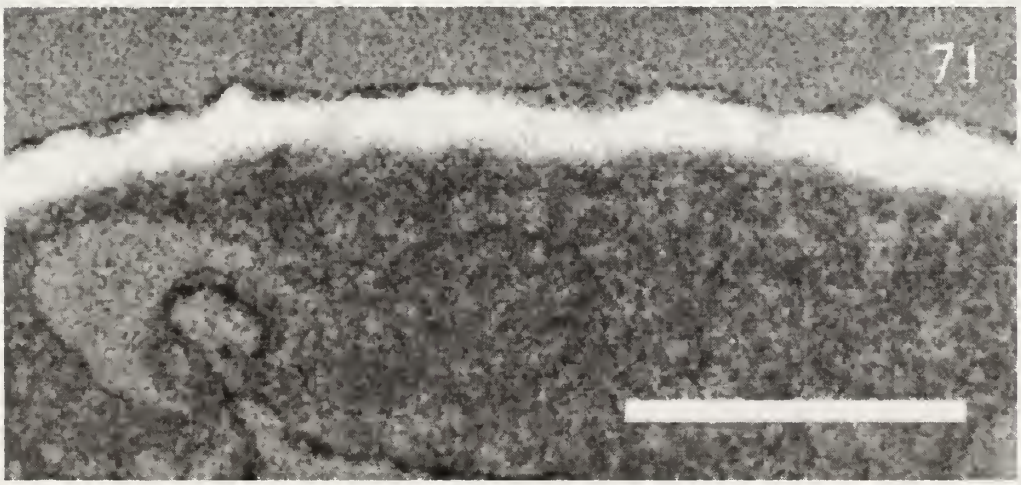


Fig. 70. Transmission electron micrograph showing localization of chitin with tomato lectin on cross section of *Achlya bisexualis* hypha. Bar=0.5 µm.

Fig. 71. Transmission electron micrograph showing the absence of chitin labeling on the cross sections of *Achlya bisexualis* hyphae resulting from preincubation of the labeling solution with re-acetylated glycol chitozan. Bar=1.00 µm.

CHAPTER 7
CONCLUSIONS

The results of cellulose localization suggest that in non-elongating hyphae, cellulose is evenly distributed along the hypha and is present in the apex. Elongating hyphae lack cellulose at the apices or there is a gradual decrease of cellulose amount toward the apices. On the other hand, the major matrix component of the wall, 1,3-ß-glucan, is distributed evenly over the elongating and non-elongating hyphae and is present in their apices. Such distribution of these two major components of *Achlya* cell wall suggests that in the elongation zone, 1,3-ß-glucans are synthesized first and cellulose deposition follows. This contradicts the idea shared by the major theories of hyphal tip growth, that all the wall components are present in the elongation zone.

The small diameters of the cellulose unlabeled regions of elongating hyphae suggest that cellulose deposition takes place almost immediately after the start of 1,3-ß-synthesis. The plastic wall that consists mainly of 1,3-ß-glucans and lacks cellulose support is stretched under the turgor pressure and/or perhaps pressure of cytoskeleton. The quickly following cellulose deposition helps to maintain the tubular cell shape and prevents the

elongation regions from "blowing out" in balloon-like structures. Cellulose is thought to provide mechanical support for the cell wall. The initial cellulose hydrolysis by cellulase in the growing apex is possible. This could create new primers in existing cellulose chains, as suggested by Maclachlan (1976) for higher plants. It was found that the growing colonies of *Achlya* secrete endocellulase (Thomas and Mullins 1967; 1969). The authors suggested that endocellulase is important for the wall softening since this fungus does not use cellulose in nutrition. The recent evidence that activity of the secreted endocellulase correlates with the tensile strength of the apical hyphal wall support this idea (Money and Hill 1997).

The results of cellulose and 1,3-ß-glucans distribution in the apical wall, combined with the results of hyphal growth monitoring suggest a new aspect of the hypothesis for hyphal tip growth. This hypothesis would state that in the *Achlya* growth process, all hyphae go through periods of elongation and no-elongation (dormancy). The elongation is not a steady process as it is generally assumed. It consists of alternating periods with fast and slow growth rates. Elongation starts with synthesis of 1,3-ß-glucans, which is quickly followed by synthesis of cellulose.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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